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TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE (DO/US)-- ENTRY INTO THE U.S. NATIONAL STAGE UNDER CHAPTER I

PCT/JP99/05156	26 October 1999	26 October 1998
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

IDENTIFICATION OF NOVEL SUBSTRATE I-TRAF OF IKK-i KINASE
TITLE OF INVENTION

Shizuo AKIRA and Takahiro SHIMADA
APPLICANT(S)

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: DO/US

NOTE: The completion of those fling requirements that can be made at a time later than 20 months from the priority date results from the Commissioner exercising his judgment under the authority granted under 35 U.S.C. § 371(d). The filing receipt will show the actual date of receipt of the last item completing the entry into the national phase. See 37 C.F.R. § 1.491, which states: "An international application enters the national stage when the applicant has filed the documents and fees required by 35 U.S.C. § 371(c) within the periods set forth in § 1.494 and § 1.495."

WARNING: *Where the items are those that can be submitted to complete the entry of the international application into the national phase subsequent to 20 months from the priority date, the application is still considered to be in the international stage. And if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (because international application papers are not covered by an ordinary certificate of mailing. 37 C.F.R. § 1.8(2)(xi)).*

WARNING: *Documents and fees must be clearly identified as a submission to enter the national stage under 35 U.S.C. § 371, otherwise the submission win be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).*

CERTIFICATION UNDER 37 C.F.R. § 1.10*
*(Express Mail label number is **mandatory.**)*
(Express Mail certification is optional.)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date **June 24, 2000**, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number **EK493794187US**, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Deanna M. Rivernider
(type or print name of person mailing paper)

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WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

WARNING: *Failure to pay the national fee within 20 months from the priority date will result in the abandonment of the application. The time for payment of the basic fee is not extendable. M.P.E.P. § 1893.01(a)(1), 6th ed., rev. 3.*

1. Applicant herewith submits to the United States Designated Office (DO/US) the following items under 35 U.S.C. 371:

a. [X] This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).

b. [X] The U.S. National Fee (35 U.S.C. § 371(c)(1)) and

[X] other fees (37 C.F.R. § 1.492), as indicated below:

2. Fees

CLAIMS FEE	**(1) FOR**	**(2) NUMBER FILED**	**(3) NUMBER EXTRA**	**(4) RATE**	**(5) CALCULATIONS**
*	**TOTAL CLAIMS**	**10 - 20 =**	**0**	**x$ 18.00=**	**$0**
	INDEPENDENT CLAIMS	**1 - 3 =**	**0**	**x$ 78.00=**	**$0**
	MULTIPLE DEPENDENT CLAIMS(S) (if applicable) + $260.00				**$ 260.00**
BASIC FEE**	**The international search fee, as set forth in § 1.445(a)(2) to be paid to the US PTO acting as an international Searching Authority:** **[ ] has been paid (37 CFR 1.492(a)(2))..... $760.00** **[ ] has not been paid (37 CFR 1.492(a)(3))..... $970.00** **[X] where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) $840.00**				**$ 840.00**
	Total of above Calculation				**= $1,100.00**
SMALL ENTITY	**Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed also. (note 37 CFR 1.9, 1.27, 1.28)**				**-**
	Subtota				**$1,100.00**
	Total National Fe				**$1,100.00**
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item 10 below). See attached "ASSIGNMENT COVER SHEET (37 CFR 3.34)".				**$**
TOTAL	**Total Fees enclose**				**$1,100.00**

***WARNING:* *"To avoid abandonment of the application, the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 20 months from the priority date; *** (2) the basic national fee (see § 1.492(a)). The 20-month time limit may not be extended." 37 C.F.R. § 1.494(b).*

i [X] A check in the amount of $ 1,100.00 to cover the above fees is enclosed.
ii. [ ] Please charge Account No. _____ in the amount of $ _____.
A duplicate copy of this sheet is enclosed.

WARNING: *If the translations of the international application and/or oath or declaration have not been submitted by the applicant within twenty (20) months from the priority date, the applicant will be so notified and given a period of time within which to file the translation and/or oath or declaration in order to prevent abandonment. The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than twenty (20) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than twenty (20) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 will apply. 37 C.F.R. § 1.494(c).*

3. A copy of the International application as filed (35 U.S.C. § 371(c)(2)):
a. [X] is transmitted herewith.
b. [ ] is not required, as the application was filed with the United States Receiving Office.
c. [ ] has been transmitted
i. [ ] by the International Bureau. Date of mailing of the application Prom form PCT/IB/308): _____.
ii. [ ] by applicant on _____.
Date

NOTE: *Section 1.494(b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 20 months from the priority date to avoid abandonment. "The International Bureau nominally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies the applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage and applicant has received notice from the International Bureau, applicant need only pay the basic national fee by 20 months from the priority date." [This can now be paid subsequently with a surcharge.] Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35.*

4. A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):
a. [X] is transmitted herewith.
b. [ ] is not required as the application was filed in English.
c. [ ] was previously transmitted by applicant on _____.
Date

5. [ ] Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE: *The Notice of January 7, 1993 indicates that 37 C.F.R. § 1.494(d) was "amended to clarify the existing practice that PCT Article 19 Amendments must be submitted by 20 months from the priority date, which time may not be extended." This Notice further advises: "Of course, the failure to do so does not result in loss of the subject matter of PCT Article 19 amendments. The applicant may submit that subject matter in a preliminary amendment filed under Section 1.121. In many cases, filing an amendment under Section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 35. See item 11(c) below. See also 37 C.F.R. § 1.494(d).*

a. [ ] are transmitted herewith.
b. [ ] have been transmitted

i. [ ] by the International Bureau. Date of mailing of the amendment (from form PCT/IB/308): _____.

ii. [ ] by applicant on _____. Date

c. [X] have not been transmitted, as

i. [ ] no notification has been received that the International Search Authority has received the Search Copy.

ii [ ] the Search Copy was received by the International Searching Authority, but the Search Report has not yet been issued. Date of receipt of Search Copy prom form PCT/ISA/202):_____.

iii. [X] applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210): 24/11/98

iv. [ ] the time limit for the submission of amendments has not yet expired. The amendments, or a statement that amendments have not been made, will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. [X] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)):

a. [ ] is transmitted herewith.
b. [ ] is not required as the amendments were made in the English language.
c. [X] has not been transmitted for reasons indicated at point 5(c) above.

7. [X] An oath or declaration of the inventor including power of attorney (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115

a. [ ] was previously submitted by applicant on _____ Date

b. [ ] is submitted herewith, and such oath or declaration

i. [ ] is attached to the application.

ii. [ ] identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or (c) and 5(b); and states that they were reviewed by the inventor, as required by 37 C.F.R. § 1.70.

iii. [X] will follow.

II. Other document(s) or information included:

8. [X] An International Search Report or Declaration under PCT Article 17(2)(a):

a. [X] is transmitted herewith.
b. [ ] has been transmitted by the International Bureau. Date of mailing from form PCT/IB/308): _____.
c. [ ] is not required, as the application was searched by the United States International Searching Authority.
d. [ ] will be transmitted promptly upon request.
e. [ ] has been submitted by applicant on _____. Date
f. [ ] is not transmitted, as the international search has not yet issued.

9. [X] An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:
   a. [X] is transmitted herewith.
      Also transmitted herewith is (are)
      [X] Form PTO-1449 (PTO/SB/08A and 08B)
      [X] Copies of citations listed
   b. [ ] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).
   c. [ ] was previously submitted by applicant on _____.
      Date

10. [ ] An assignment document is transmitted herewith for recording. A separate
    [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or
    [ ] FORM PTO—1595 is also attached.
    [ ] Please mail the recorded assignment document to:
       i. [ ] the person whose signature and address appears below.
       ii. [ ] the following:

11. [X] Additional documents
    a. [X] Copy of request (PCT/RO/101)
    b. [X] International Publication No. WO 00/24908
       i. [X] Specification, claims and drawing
       ii. [ ] Front page only
    c. [ ] Preliminary amendment (37 C.F.R. § 1.121)
    d. [ ] Other: Form PCT/IPEA/401
       Form PCT/IPEA/409

12. [X] The above checked items are being transmitted
    a. [ ] before the 18th month publication.
    b. [X] after publication and the article 20 communication, but before 20 months from the priority date.
    c. [ ] after 20 months (revival).

NOTE: Petition to revive (37 C.F.R. § 1.137(a) or (b)) is necessary if 35 U.S.C. § 371 requirements are submitted after 20 months.

13. [ ] Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____ namely:
    Date

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 04-1105.

[X] 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 20 months without extension (37 C.F.R. § 1.494(b)(2)), results in abandonment of the application, it would be best to always check the above box.*

[X] 37 C.F.R. § 1.492(b), (c), and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment, prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

[X] 37 C.F.R. § 1.17 (application processing fees)
[ ] 37 C.F.R. § 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).

[ ] 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b)).

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying or at the time of paying . . . issue fee...." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[ ] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 20 months after the priority date.

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Docket No. 49862

U.S. PATENT APPLICATION

Title: **IDENTIFICATION OF NOVEL SUBSTRATE I-TRAF OF IKK-i KINASE**

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IDENTIFICATION OF NOVEL SUBSTRATE I-TRAF OF IKK-i KINASE

Technical Field

The present invention relates to a novel IκB kinase, the gene for it and a pharmaceutical composition containing it. More particularly, the present invention pertains to a novel IκB kinase, IKK-i, which is a novel serine/threonine kinase capable of activating transcription factor NF-κB which regulates expression of various genes involved in immune response and binds with I-TRAF to phosphorylate it.

Background Art

Macrophages play an important role in biological defense mechanisms and are known to play major roles in functions such as phagocytosis. They are also involved in antigen presentation against bacterial infections and infiltration by malignant tumor. Further, macrophages are activated by lipopolysaccharide (LPS) and inflammatory cytokines, and they express various genes involved in the immune response. These include the major histocompatibility antigen, TNF-α (tumor necrosis factor-α), IL-1β (interleukin-1β), IL-6 (interleukin-6) and MIP-1α/β (macrophage inflammatory protein-1α/β).

NF-κB is a transcription factor which regulates the expression of various genes involved in the immune response. NF-κB is known to be activated by LPS, TNF-α and IL-1β, and to regulate transcription of genes of TNF-α, IL-1β and IκB-α, which have important roles in the immune response.

In order to identify a novel gene involved in the immune response, the inventors have performed subtractive hybridization between a (+) group and a (-)

group of LPS stimulation from the macrophage tumor strain, RAW 264.7. The gene obtained in this way, clone #2F9, was demonstrated to be a novel gene having homology with IκB kinase-α,β (DiDonato, J.A., et al., Nature 1997, Aug. 7, 388 (6642), 548-554; Zandi, E. et al., Cell 1997, Oct. 17, 91(2), 243-252; Mercurio, F. et al., Science 1997, Oct. 31, 278 (5339), 860-866; Woronicz, J.D., et al., Science 1997, Oct. 31, 278 (5339), 866-869; Regnier, C.H., et al., Cell 1997, Jul. 25, 90(2), 373-383). This gene was identified recently and is known to activate NF-κB. The inventors have given the name IKK-i (inducible-IκB kinase) to the protein coded by this novel gene.

Disclosure of Invention

The present invention provides a novel IκB kinase, IKK-i, which is a novel serine/threonin kinase capable of activating transcription factor NF-κB which regulates expression of various genes involved in immune response, and also the gene coding for the same and pharmacyeutical composition containing the same.

The inventors have proved that IKK-i is a novel serine/threonine kinase which phosphorylates IκB and activates NF-κB, and whose expression is induced by various inflammatory cytokines.

The present invention relates to a protein having an amino acid sequence represented by SEQ ID NO: 2 or SEQ ID NO: 4, or an amino acid sequence in which one or more amino acids in the said amino acid sequence is deleted or substituted by other amino acids and/or one or more other amino acid is added, and being able to activate the transcription factor NF-κB. The protein of the present invention is a novel serine/threonine kinase.

The present invention further relates to a gene having a base sequence which codes for the above mentioned novel protein. More particularly, the present invention

pertains to a gene having a base sequence represented by SEQ ID NO: 1 or SEQ ID NO: 3.

The present invention more further relates to a pharmaceutical composition comprising the above mentioned protein and a pharmaceutically acceptable carrier thereof. The pharmaceutical composition of the present invention can activate the transcription factor NF-κB and also acts on the immune response mechanism. Further, the pharmaceutical composition of the present invention is useful as a preventive or therapeutic agent against diseases involving I-TRAF or TRAF molecule.

Brief Description of Drawings

Fig. 1 is a drawing replaced by a photograph showing the results of northern blotting analysis, in which the induction of expression of mRNA of IKK-i before stimulation (-) or after stimulation (+) by LPS is shown. The lower photograph shows the results obtained using G3PDH.

Fig. 2 is a drawing replaced by a photograph showing the results of northern blotting analysis, in which expression of mRNA of IKK-i after LPS stimulation is shown in a time-dependent manner. The lower photograph shows the results obtained using G3PDH.

Fig. 3 shows a comparison of the amino acid sequences of human IKK-i and mouse IKK-i. In the figure, the part enclosed by a rectangle shows an identical sequence; a part [ ] shows a kinase domain; and * shows a leucine-zipper domain

Fig. 4 shows a comparison of the amino acid sequences of IKK-i, IKK-α and IKK-β. The parts colored with a gray backgrounds in the figure show identical sequences; the part [ ] shows a kinase domain; the part enclosed by a rectangle shows an activation loop; and * mark shows an amino acid residue which may be important

for kinase activity.

Fig. 5 is a drawing replaced by a photograph showing the expression of IKK-i in various organs based on northern blotting analysis.

Fig. 6 is a drawing replaced by a photograph showing the expression of IKK-i in B cells and T cells based on northern blotting analysis. The lower photograph shows result of using G3PDH.

Fig. 7 is a drawing replaced by a photograph showing the expression of IKK-i in a mouse tumor strain based on northern blotting analysis. The lower photograph shows total RNA stained with ethidium bromide.

Fig. 8 shows a drawing replaced by a photograph showing the induction of IKK-i in mouse peritoneal macrophages as a result of various stimulations based on northern blotting analysis. The lower photograph shows the result obtained using G3PDH.

Fig. 9 shows the results of activation of the NF-κB reporter gene by enforced expression of IKK-i. The lower photograph is a drawing replaced by a photograph, showing the results of an evaluation of the amount of protein. These results were obtained by immunoblotting using anti-FLAG antibody (M2).

Fig. 10 is a drawing replaced by a photograph showing the results of phosphorylation of IκB-α by IKK-i in vitro. The lower photograph shows the results of evaluation of the amount of protein based on the results of immunoblotting using anti-FLAG antibody (M2).

Fig. 11 is a drawing replaced by a photograph showing the formation of complexes of IKK-i and I-TRAF in cells. In Fig. 11, lane 1 shows Flag-IKK-I, lane 2 shows Myc-I-TRAF, and lane 3 shows Flag-IKK-i and Myc-I-TRAF respectively.

Fig. 12 is a drawing replaced by a photograph showing the binding region of

IKK-i obtained by using an I-TRAF deletion mutant. In Fig. 12, lane 1 shows 1 - 170 fragment, lane 2 shows 1 - 247 fragment, lane 3 shows 193 - stop fragment, and lane 4 shows full length (FL) respectively.

Fig. 13 is a drawing replaced by a photograph showing the phosphorylation of I-TRAF by IKK-i. In lanes 1, 3 and 5 in Fig. 13, only Flag-IKK-i is transfected, and in lanes 2, 4 and 6, both of IKK-i and I-TRAF are transfected.

Fig. 14 is a drawing replaced by a photograph showing the phosphorylation region of I-TRAF. In Fig. 14, lanes 1 and 5 show 1 - 170 fragment, lanes 2 and 6 show 1 - 247 fragment, lanes 3 and 7 show 193 - stop fragment, and lanes 4 and 8 show full length (FL) respectively.

Fig. 15 is a drawing replaced by a photograph showing the phosphorylation of purified GST-I-TRAF by IKK-i. In Fig. 15, lane 1 shows that Flag-IKK-i is transfected, and lane 2 shows that the mutant IKK-i (K38A) is transfected.

Best Embodiment for Carrying Out the Invention

Firstly, cDNA cloning of IKK-i of the present invention is explained.

Using the suppression subtractive hybridization technique, a subtraction between a group with lipopolysaccharide (LPS) stimulation (+) and a group without LPS stimulation (-) was performed against RAW 264.7, a macrophage tumor strain. Subsequently, screening for the gene induced by LPS stimulation was carried out. Seven new gene fragments, in addition to known genes such as MIP-1α/β, G-CSF and TNF-α, were obtained.

As the result of testing these fragments, only slight expression of clone #2F9 was observed in RAW 264.7 without stimulation, but the expression was markedly increased after 4 hours of the LPS stimulation (refer to Fig. 1). Fig. 1 shows the

results of northern blotting analysis of the gene induced by lipopolysaccharide (LPS) stimulation of macrophage tumor strain RAW 264.7. Samples containing poly(A)$^+$RNA 2μg before (-) or after (+) LPS (100 ng/ml) stimulation of RAW 264.7 were electrophoresed in 1% formamide-agarose and transferred to nylon membranes; they were then hybridized using a probe containing a cDNA fragment (2Fa) of IKK-i obtained by the subtraction. Fig. 1, lower photograph, shows that the amount of RNA is equivalent to the amount obtained by using G3PDH.

As for the time course of expression of the mRNA, it started to increase 2 hours after from LPS stimulation, reached a peak value after 4 hours, and returned to the original level after 24 hours (refer to Fig. 2).

Fig. 2 shows that the results based on northern blotting analysis are similar to those in Fig. 1. Namely, RAW 264.7 was stimulated by 100 ng/ml LPS, the total RNA was extracted after the indicated number of hours in each lane, then 25 μg of each sample was electrophoresed in 1% formamide-agarose. The coding region of mouse IKK-i (mIKK-i) was used as a probe. In Fig. 2, the lower photograph shows that the amount of RNA is equivalent to that obtained by using G3PDH.

A cDNA library was prepared using mRNA obtained by LPS stimulation of RAW 264.7 for 4 hours, and the full length of this gene was obtained by using fragment 2F9 as a probe. This gene codes for 718 amino acids in a 2154 bp open reading frame. As a result of a homology search, a human cDNA clone KIAA 0151, for which the base sequence was determined but the function was not known, showed the highest homology in the database. The homology was 82.3% in the amino acid level, consequently 2F9 was thought to be a counterpart of KIAA 0515 in the mouse.

The gene showing the second highest homology next to KIAA 0151 was IκB kinase-α, β (IKK-α and IKK-β), which has been recently identified and

demonstrated to phosphorylate IκB-α and to activate NF-$\kappa\beta$. The homology of the kinase domain is 29.1% and 30.1% in the amino acid level, respectively.

2F9 and KIAA have been confirmed to have a serine/threonine kinase domain in the N-terminal end, and a leucine-zipper domain in the center. Consequently, based on the similarities of the structures of IKK-α and IKK-β and the function of these molecules, for which more explanation will be given later, the inventors have named this novel kinase as inducible IKK (IKK-i).

Comparisons of the amino acid sequences of human IKK-i and mouse IKK-i are shown in Fig. 3, and comparisons of the amino acid sequences of human IKK-i, human IKK-α and human IKK-β are shown in Fig. 4.

In Fig. 3, the identical sequences are enclosed by rectangles and the kinase domain is indicated by [ ]. The leucine-zipper domain is indicated with an * mark below the domain.

In Fig. 4, the backgrounds of the identical sequences are colored with gray and the kinase domain is indicated by [ ]. The activation loop is enclosed by a rectangle. The amino acid residues, which are thought to be important for kinase activity in the activation loop sequence, are indicated with an * mark. Under the helix loop - the helix structures of IKK-α and IKK-β are shown by underlining.

The expression of IKK-i in various organs was analyzed by northern blotting. IKK-α and IKK-β were generally expressed in all tissues, on the contrary, mRNA of IKK-i was primarily expressed specifically in spleen, thymus, peripheral leukocytes, pancreas and placenta (refer to Fig. 5). Northern blotting analysis was performed by using multi-tissue northern blot membranes (Clontech), in which 2 μg of poly $(A)^+$RNA obtained from each of the indicated organs was placed in each lane shown in Fig. 5. A human IKK-i (hIKK-i) coding region was used as a probe.

In order to determine from which cell population the expression of IKK-i found in the spleen originated, B-cells and T-cells were isolated by using anti-B220 antibody. Before (-) and after (+) LPS stimulation of B-cells, and before (-) and after (+) phorbol ester and calcium ionophore stimulations of T-cells, the expression of IKK-i was analyzed by northern blotting. Though the expression was not able to be detected in B-cells without LPS stimulation, the expression was induced by LPS stimulation.

Constitutive expression was observed in T-cells, but stimulation by phorbol ester and calcium ionophore reduced the expression (refer to Fig. 6).

Fig. 6 shows the results of northern blotting analysis carried out as follows. B-cells and T-cells were isolated from the spleen cells collected from C57BL/6 using antibody (B220) in a high-gradient magnetic cell separation system MACS (Miltenyi Biotec, Berg.-Gladbach, Germany). B-cells were stimulated by 100 μg/ml of LPS 100 and T-cells were stimulated by 10 μM of ionomycin and 10 μg/ml of PMA, each for 4 hours. Before and after the stimulation, the total RNA was extracted, and 20 μg of each sample was electrophoresed using 1% formamide agarose, and analyzed by northern blotting in the same way as the case in Fig. 2. The coding region of mouse IKK-i (mIKK-i) was used as a probe. In Fig. 6, the lower photograph shows that the amount of RNA obtained is equivalent to that obtained by using G3PDH.

The expression of IKK-i in mouse cell strains was analyzed before (-) or after (+) stimulation. The expression of IKK-i was induced in 5E3 (a natural killer cell clone) and M1 (a monocytic leukemia cell line) by stimulation with LPS (refer to Fig. 7).

The total amounts of RNA before (-)and after (+) 4 hours of stimulation with LPS (100 ng/ml) were extracted from tumor strain NIH 3T3 (a fibroblast cell line),

EL-4 (thymoma cells), 5E3 (a natural killer cell clone), MOPC 315 (myeloma cells), BCL-1 (B cell leukemia cells) and M1 (a monocytic leukemia cell line) shown in each lane in Fig. 7, and analyzed by northern blottings using the same methods as shown in Fig. 2. The results are shown in Fig. 7. The coding region of mIKK-i was used as a probe. The lower part of Fig. 7 shows that the amount of RNA is equivalent to that obtained using the electrophoretic patterns of total RNA stained with ethidium bromide.

A further enhancement effect of stimulation, other than the effect of LPS on expression of IKK-i, was examined. Peritoneal macrophages collected from C57BL/6 were stimulated by LPS, PMA, TNF-α, IL-1β, IFN-γ or IL-6 for 4 hours, and the expression of IKK-i was observed. IKK-i was induced by TNF-α, IL-1β, IFN-γ or IL-6 instead of LPS, but was not induced by PMA (refer to Fig. 8).

Fig. 8 shows that the peritoneal macrophages collected from C57BL/6 were stimulated by LPS: 1 μg/ml, PMA: 10 ng/ml, RNF-α: 100 ng/ml, IL-1β: 100 ng/ml, IFN-γ: 250 U/ml or Il-6: 2000 U/ml as shown in the upper part of each lane in Fig. 8. Subsequently, the total RNA was extracted then analyzed by northern blottings in the same way as shown in Fig. 2. The coding region of mIKK-i was used as a probe. The lower part of Fig. 8 shows that the amount of RNA obtained is equivalent.

It has been proved by a reporter gene assay that enforced intracellular expression of IKK-α and IKK-β results in activating NF-κB. Owing to the structural similarities of IKK-i to them, the possibility of activating NF-κB might be expected. Consequently, the possibility of NF-κB activity of IKK-i was examined by a reporter gene assay.

First, a construct (pEF-BOS-FLAG-WT-IKK-i), in which FLAG epitope was tagged onto the N-terminal of IKK-i and inserted into pEF-BOS expression vector,

was prepared. Then, pEF-BOS-FLAG-IKK-i or control of a vector only were transiently cotransfected into the luciferase reporter construct of NF-κB and 293T cells, and luciferase activities were assayed. As the result, it was demonstrated that IKK-i activated NF-κB in an expression-dependent manner (refer to Fig. 9).

Fig.9 shows the results of the luciferase activity assay. In these studies, 293T cells were transiently cotransfected with the reporter construct (pNF-κB -Luc) in which luciferase gene was ligated with the NF-κB consensus sequence, the construct (pEF-BOS-FLAG-WT-IKK-i) in which FLAG epitope was tagged onto the N-terminal of IKK-i gene and subcloned into pEF-BOS expression vector, or a control of vector only. The total amounts of DNA were regularized to 4 μg by using a pEF-BOS vector. The amounts of transfected pEF-BOS-FLAG-WT-IKK-i are shown in the lower part of the graph, and the lower part of Fig. 9 shows the amount of protein, which was determined by immunoblotting using anti-FLAG antibody (M2).

IKK-α and IKK-β have been known to phosphorylate IκB-α in vitro. It was analyzed by an in vitro kinase assay whether or not the IKK-i of the present invention was able to phosphorylate the serine residues at No. 32 and No. 36.

A mutant construct (pEF-BOS-FLAG-K38A-IKK-i), which was prepared by mutating the No. 38 lysine of pEF-BOS-FLAG-WT-IKK-i or IKK-i to alanine, was transiently transfected into the 298T-cells. The IKK-i protein or the K38A-IKK-i protein expressed after 24 hours was purified by immunoprecipitation with anti-FLAG antibody (M2) and was used for an in vitro kinase assay.

GST-IκB-αN protein (WT) which was prepared by removing the C-terminal from an ankyrin repeat of IκB-α, or GST- IκB-αN protein (AA) which was prepared by replacing both of the No. 32 and No. 36 serine residues of GST-IκB-αN with alanine, was used as a substrate. A band of autophosphorylated IKK-i at about 80 kDa was

observed in a lane of the BOS-FLAG-WT-IKK-i. Though IKK-i phosphorylated GST-I κB-αN protein (WT), K38A-IKK-i did not phosphorylate GST-IκB-αN protein (WT). Further, IKK-i did not phosphorylate GST-IκB-αN protein (AA)(refer to Fig. 10).

Fig. 10 shows the results of phosphorylation of IκB-α by IKK-i in vitro. A pEF-BOS-MOCK, a pEF-BOS-FLAG-WT-IKK-i, or a mutant construct (pEF-BOS-FLAG-K38A-IKK-I) which was prepared by mutating the No. 38 lysine of pEF-BOS-FLAG-WT-IKK-i to alanine, was transiently transfected into the 298T-cells. The IKK-i protein or the K38A-IKK-i protein expressed after 24 hours was purified by anti-FLAG antibody (M2) using immunoprecipitation, and was used for an in vitro kinase assay. GST-IκB-αN protein (WT) which was prepared by removing the C-terminal from an ankyrin repeat of IκB-α, or GST- IκB-αN protein (AA) which was prepared by replacing both of the No. 32 and No. 36 serine residues of GST-IκB-αN with alanine, was expressed in E. coli and purified using glutathione Sepharose, and the resulting products were used as substrates. IKK-i or K38A-IKK-i as substrates and [γ-^{32}P]ATP were reacted at 30℃ for 20 minutes. The reaction mixture was developed with SDS-PAGE and measured by autoradiography. Arrows point to the bands of autophosphorylation and GST-IκB-αN. The molecular weight (kDa) is shown on the left side. The lower part of Fig. 10 shows the results of determination of the amount of protein by immunoblotting using anti-FLAG antibody (M2).

As a result, it was elucidated that the IKK-i of the present invention phosphorylated a serine residue in the N-terminal of IκB which played an important role in the activation of NF-κB.

The base sequences and amino acid sequences clarified as a result of cDNA cloning of the IKK-i of the present invention are shown in the sequence listings. SEQ

ID NO: 1 shows the base sequence for human IKK-i (hIKK-i) and SEQ ID NO: 2 shows the amino acid sequence for hIKK-i. SEQ ID NO: 3 shows the base sequence for mouse IKK-i (mIKK-i) and SEQ ID NO: 4 shows the amino acid sequence for mIKK-i.

It was found that the IKK-i of the present invention was a novel serine/threonine kinase having a kinase domain in the N-terminal and a leucine-zipper domain in the center and the expression of its mRNA was induced by LPS stimulation of macrophages. The amino acid sequence of the IKK-i of the present invention showed high homologies with IKK-α and IKK-β, which phosphorylate IκB and activate NF-κB.

The IKK-i of the present invention was expressed constitutively in the spleen, thymus and peripheral leukocytes, and was also expressed constitutively in T-cells in the spleen. The expression was enhanced by LPS stimulation of B-cells, peritoneal macrophages, natural killer cells, and a monocyte tumor strain, and was also enhanced by stimulation of peritoneal macrophages by TNF-α, IL-1β, IFN-γ or IL-6. As a result of northern blotting analysis, it has been shown that expression of IKK-i is directed mainly to immune competent cells and cells involved in the inflammatory reaction, and IKK-i may be a molecule involved in the inflammatory reaction since it is enhanced by inflammatory stimulation.

Activation ability of IKK-i for NF-κB was analyzed by a reporter gene assay, and it was found that enforced expression of IKK-i in the 293T cells resulted in activating NF-κB in a amount-of-protein-dependent manner. From the fact that IKK-i can phosphorylate a serine residue in the N-terminal of IκB-α in a manner similar to the actions of IKK-α and IKK-β, as elucidated by an in vitro kinase assay, it is suggested that the activation ability for the NF-κB observed in the reporter gene assay may be dependent on phosphorylation of the N-terminal of IκB-α by IKK-i.

IKK-i is a novel IκB kinase, expression of which is induced by inflammatory stimulation of immune competent cells, and which activates NF-κB.

The IKK-i of the present invention may possibly have the ability to contribute to maintaining the activation of NF-κB by stimulation of the LPS. This is suggested by the facts that the amount of expression increases 2 hours after from LPS stimulation and NF-κB is activated in an expression-dependent manner. Since expression of IKK-i is directed to the immune competent cells as compared with IKK-α and IKK-β, the development of an inhibitor may possibly to be able to suppress the activation of NF-κB specific to the immune system. Consequently, controlling IKK-i may contribute to the treatment of inflammatory diseases.

Further, in order to isolate a molecule which interacts with the IKK-i of the present invention, a yeast two hybrid method was performed. Amino acids No. 541 - 716 from human IKK-i were inserted into a plasmid, pAS2-1, for expression of a chimera protein with a GAL4 DNA binding domain in order to prepare a bait plasmid. This was then transformed to yeast Y190 and grown on a selective medium. The transformant thus obtained was further transformed with a pACT2 plasmid containing a human B-cell derived cDNA library, which was able to express the chimera protein with a GAL4 activation domain. Cells were then grown on a selective medium. The plasmid was recovered from the positive clones thus obtained. Finally the base sequence was determined by using a DNA sequencer. As a result of a homology search, 10 clones were found to be identical to the sequence I-TRAF/TANK which had been already known.

It was examined that IKK-i and I-TRAF were able to be bound to each other in cells. First, an expression vector, which was able to express in mammalian cells, was constructed. Flag was linked to the N-terminal of human IKK-i as an epitope,

and then inserted into the expression vector pEF-BOS. Myc was added to the N-terminal of human I-TRAF as an epitope, and then inserted into the expression vector pEF-BOS. These were both transfected into a monkey kidney cell line, COS-7 cells, by lipofection. After 24 hours, the cells were solubilized with buffer containing 1.0% Nonidet P-40. The resulting material was immunoprecipitated by anti-Flag antibody or anti-Myc antibody, and was then analyzed by means of western blotting using anti-Myc antibody or anti-Flag antibody.

Results are shown in Fig. 11. In Fig. 11, lane 1 shows Flag-IKK-I, lane 2 shows Myc-I-TRAF, lane 3 shows Flag-IKK-i and Myc-I-TRAF respectively. Bands including Flag-IKK-I which was immunoprecipitated with anti-Myc antibody (Fig. 11, upper part, lane 3) and Myc-I-TRAF which was coimmunoprecipitated with anti-Flag antibody (Fig. 11, lower middle parts, lane 3) were specifically detected. Consequently, both molecules were demonstrated to form complexes in the mammalian cells. The amount of expression in each lane can be confirmed by means of western blotting analysis of the immunoprecipitates of anti-Myc antibody or anti-Flag antibody with anti-Myc antibody or anti-Flag antibody (Fig. 11, upper middle and lower parts).

Next, it was determined through which domain of the I-TRAF IKK-i is bound. This was examined by using the same methods as the above. First, three types of I-TRAF defective mutants were prepared. A fragment of the amino acid sequence of amino acids No. 1 - 170, 1 - 247 or 193 - C-terminal (stop) from human I-TRAF to which Myc was added to the N-terminal thereof, was inserted into pEF-BOS. These were then transfected with Flag-IKK-i into COS-7 cells. After 24 hours the cells were solubilized. The resulting solubilized materials were immunoprecipitated with anti-Myc antibody, then analyzed by means of western blotting with anti-Flag antibody.

The results are shown in Fig. 12. In Fig. 12, lane 1 shows 1 - 170 fragment, lane 2 shows 1 - 247 fragment, lane 3 shows 193 - stop fragment and lane 4 shows FL (full length) respectively. In the expressed cells, a band of Flag-IKK-i, which is immunoprecipitated by anti-Myc antibody, is detected (Fig. 12, upper part). Consequently, it was demonstrated that I-TRAF bound with IKK-i through the 170 amino acids in the N-terminal. The amount of expressed protein in each lane was confirmed by means of western blotting analysis using anti-Flag antibody (Fig. 12, middle part) or anti-Myc antibody (Fig. 12, lower part) with the material described above.

Next, It was determined whether or not I-TRAF is a substrate for phosphorylation by IKK-i. Flag-IKK-i and Myc-I-TRAF were transfected into COS-7 cells and solubilized after 24 hours. After immunoprecipitating the resulting material with anti-Flag antibody or anti-Myc antibody, kinase buffer and [γ-^{32}P]ATP were added to the precipitate and allowed to react. Then an in vitro kinase assay was performed.

The results are shown in Fig. 13. Lanes 1, 3 and 5 show the results of transfection with only Flag-IKK-i, and lanes 2, 4 and 6 show the results of transfection with both Flag-IKK-i and Myc-I-TRAF. Bands of phosphorylated Myc-I-TRAF were detected in both of immunoprecipitates with anti-Flag antibody (lane 2) or anti-Myc antibody (lane 4). Consequently, it was clearly demonstrated that Myc-I-TRAF was a substrate which was phosphorylated by IKK-i. The amounts of expression of protein in each lane were confirmed by means of western blotting analysis of the products of solubilizing with anti-Flag antibody (Fig. 13, upper parts of lanes 5 and 6) or anti-Myc antibody (Fig. 13, lower parts of lanes 5 and 6).

Next, it was examined which region of I-TRAF was required for

phosphorylation. Myc-I-TRAF defective mutants [a fragment of amino acids No. 1 - 170, a fragment of amino acids No. 1 - 247, a fragment of amino acids No. 197 - stop and full length (FL)] were transfected with IKK-i into COS-7 cells and the resulting solubilized material was obtained. After that, immunoprecipitation was performed with anti-Flag antibody or anti-Myc antibody and an in vitro assay was performed.

The results are shown in Fig. 14. In Fig. 14, lanes 1 and 5 show a fragment of amino acids No. 1 – 170, lanes 2 and 6 show a fragment of amino acids No. 1 – 247, lanes 3 and 7 show a fragment of amino acids No. 197 - stop and lanes 4 and 8 show full length (FL) respectively. As shown, phosphorylations of FL (lanes 4 and 8) and Myc-I-TRAF (1-247)(lanes 2 and 6) were confirmed. Consequently, the region, where the phosphorylation by IKK-I occurs, may be located at least within amino acids No. 171 - 247 in the amino acid sequence of I-TRAF.

Further, it was examined whether or not I-TRAF was phosphorylated by IKK-i by using the purified protein of I-TRAF. First, the I-TRAF protein was purified. Human I-TRAF cDNA was then inserted into an expression vector pGEX-5X-i, which was able to be expressed as a chimera protein with glutathione S transferase (GST) in E. coli. The vector thus obtained was transformed into E. coli DH5α. After E. coli was cultured overnight in LB liquid medium, IPTG was added and the mixture was incubated for 3 hours. The collected E. coli cells were solubilized by PBS and disrupted by ultrasonication. Triton X-100 was added up to 1% to solubilize the mixture, glutathione Sepharose was added and the mixture was allowed to react for 1 hour. GST-I-TRAF protein bound with glutathione Sepharose was eluted with a glutathione solution and used as the purified protein for the experiment. Flag-IKK-i was transfected into COS-7 cells. Simultaneously, a mutant IKK-i (K38A) was prepared and transfected. In the K38A mutant, the No. 38 lysine in the kinase

domain, which is thought to be an ATP binding site, is substituted by alanine. It is known that kinase activity is lost by a mutation at this site in many kinases. After 24 hours of transfection, cells were solubilized and immunoprecipitated with anti-Flag antibody. A purified GST-I-TRAF (1.0 μg) was added to the immunoprecipitate and phosphorylated in vitro to perform a kinase assay.

The results are shown in Fig. 15. In Fig. 15, lane 1 shows the results of transfection with Flag-IKK-i and lane 2 shows results of transfection with mutant IKK-i (K38A). As shown, in cells in which Flag-IKK-i was expressed, phosphorylation of GST-I-TRAF was observed (Fig. 15, upper part of lane 1). No phosphorylation was observed with the K38A mutant (Fig. 15, upper part of lane 2). Consequently, it was elucidated that I-TRAF was a substrate which was phosphorylated by IKK-i. Further, intracellular expression of the K38A was confirmed by means of western blotting analysis of the solubilized product with anti-Flag antibody (Fig. 15, lower part).

As a result, the binding of IKK-i with I-TRAF was elucidated. Further it was shown by an in vitro kinase assay that I-TRAF was a specific substrate which was phosphorylated by IKK-i. I-TRAF was firstly identified as a molecule bound with TRAF 2 and TRAF 3. At present, six types of TRAF molecules have been identified and are known to function as adapter molecules bound with various receptors. Especially, the TRAF molecule binds with an apoptosis-related TNF receptor and CD40, and has been known as a signal transduction molecule for these receptors.

The TRAF molecule is activated by forming a complex with a receptor as a result of stimulation by a ligand. However, when activation does not occur, activation is thought to be negatively regulated by binding with I-TRAF in the cytoplasm. Consequently, IKK-i is thought to be involved indirectly in the activation of the TRAF molecule by phosphorylation of the I-TRAF.

As previously explained, since the IKK-i of the present invention is involved in activation of the TRAF molecule, which relates to apoptosis, it is useful for the prevention, treatment and regulation of apoptosis-related diseases related to the TRAF molecule.

Also as previously explained, IKK-i is thought to be a very interesting molecule from a clinical standpoint. A pharmaceutical composition of the present invention is comprised of IKK-i and pharmaceutically acceptable carrier and can be administered in the form for administration. The dosage can be adjusted according to the situation of the patients.

The pharmaceutical composition of the present invention is effective for improvement of the immune response function and for treatments for inflammatory diseases.

The present invention also includes the use of antisense against the IKK-i gene as a component of the pharmaceutical composition of the present invention.

Examples

Following examples illustrate the present invention but are not construed as limiting the invention.

Example 1 (cDNA cloning of mIKK-i)

Using the suppression subtractive hybridization technique, a subtraction between a group with 100 ng/ml of lipopolysaccharide (LPS) stimulation (+) and a group without the LPS stimulation (-) was performed against RAW 264.7, a macrophage tumor strain. Subsequently, screening for the gene induced by LPS stimulation was carried out. As a result, seven new gene fragments, in addition to

known genes such as MIP-1α/β, G-CSF and TNF-α, were obtained. Among seven novel genes, clone #2F9 was a gene fragment having 374 bp. In order to obtain full length of gene of the clone #2F9, a cDNA library was prepared using λZAP phage from mRNA obtained by LPS stimulation of RAW 264.7 for 4 hours. A fragment of 374 bp in the 2F9 was labeled with α-^{32}P-dCTP by random labeling technique. A full length gene was obtained by screening of cDNA phage library using the labeled 2F9 as a probe.

The full length of the clone thus obtained was 2910 bp. This gene codes for 718 amino acids in a 2154 bp open reading frame. The base sequence of this gene is shown in SEQ ID NO: 3. The amino acid sequence is shown in SEQ ID NO: 4.

Example 2 (cDNA cloning of hIKK-i)

Based on the previously registered base sequence KIAA0151 in DDBJ, cDNA of human IKK-i was cloned by means of PCR using human placental cDNA library as a template. The sequences of the primer used for PCR were as follows:

5'-ctttgcctgactcagggcagctcagag-3', and

5'-atggtgcagaagagcagtgttggaatc-3'

This gene coded 716 amino acids in 2148 bp open reading frame. The base sequence of this gene is shown in SEQ ID NO:1. Amino acid sequence is also shown in SEQ ID NO: 2.

Example 3 (Expression of clone #2F9 by LPS stimulation)

The northern blotting analysis of the gene induced by lipopolysaccharide

(LPS) stimulation of macrophage tumor strain RAW 264.7 was performed. Samples containing 2μg of poly(A)$^+$RNA before (-) or after (+) LPS (100 ng/ml) stimulation of RAW 264.7 were electrophoresed in 1% formamide-agarose and transferred to nylon membranes, then hybridized using a probe containing a cDNA fragment (2Fa) of IKK-i obtained by the subtraction.

Results are shown in Fig. 1. The lower photograph in Fig.1 shows that the amount of RNA is equivalent to the amount obtained by using G3PDH.

Example 4 (Time-course of expression of clone #2F9 by LPS stimulation)

RAW 264.7 was stimulated by 100 ng/ml of LPS, the total RNA was extracted after 0.5, 2, 4, 8, 12 and 24 hours, respectively, then 25 μg of each sample was electrophoresed in 1% formamide-agarose and northern blotting analysis was performed similar to those in example 3. The coding region of mouse IKK-i (mIKK-i) obtained in example 2 was used as a probe.

The results are shown in Fig. 2. The lower photograph in Fig. 2 shows that the amount of RNA is equivalent to that obtained by using G3PDH.

Example 5 (Expression of IKK-i)

The expression of IKK-i in various organs was analyzed by northern blotting.

Northern blotting analysis was performed by using multi-tissue northern blot membranes (Clontech), in which 2 μg of poly (A)$^+$RNA obtained from each organ was placed. A human IKK-i (hIKK-i) coding region was used as a probe.

Results are shown in Fig. 5. In Fig. 5, an arrow indicates a position of IKK-i.

Example 6 (Expression of IKK-i in spleen)

B-cells and T-cells were isolated from the spleen cells collected from C57BL/6 using antibody (B220) in a high-gradient magnetic cell separation system MACS (Miltenyi Biotec, Berg.-Gladbach, Germany). B-cells were stimulated by 100 μg/ml of LPS and T-cells were stimulated by 10 μM of ionomycin and 10 μg/ml of PMA, each for 4 hours. Before and after the stimulation, the total RNA was extracted, and 20 μg each of the samples were electrophoresed using 1% formamide agarose, and analyzed by northern blotting in the same way as the case in example 4. The coding region of mIKK-i was used as a probe.

Results are shown in Fig. 6. In Fig. 6, the lower photograph shows that the amount of RNA obtained is equivalent to that obtained by using G3PDH.

Example 7 (Expression of IKK-i in cells of mice)

The total amounts of RNA before (-) and after (+) 4 hours of the stimulation with LPS (100 ng/ml) were extracted from tumor strain NIH 3T3 (a fibroblast cell line), EL-4 (thymoma cells), 5E3 (a natural killer cell clone), MOPC 315 (myeloma cells), BCL-1 (B cell leukemia cells) and M1 (a monocytic leukemia cell line) of mouse cell strains, and analyzed by northern blottings using the same methods as shown in example 4. The coding region of mIKK-i was used as a probe.

The results are shown in Fig. 7. The lower part of Fig. 7 shows that the amount of RNA is equivalent to that obtained using the electrophoretic patterns of total RNA stained with ethidium bromide.

Example 8 (Expression of IKK-i in peritoneal macrophages)

The peritoneal macrophages collected from C57BL/6 were stimulated by LPS: 1 μg/ml, PMA: 10 ng/ml, RNF-α: 100 ng/ml, IL-1β: 100 ng/ml, IFN-γ: 250 U/ml or

Il-6: 2000 U/ml. Subsequently, the total RNA was extracted then analyzed by northern blottings in the same way as in example 4. The coding region of mIKK-i was used as a probe.

Results are shown in Fig. 8. In Fig. 8, (-) indicates a case without stimulation.

The lower part of Fig. 8 shows that the amount of RNA obtained is equivalent to that obtained by using G3PPH.

Example 9 (Construction of IKK-i expression vector)

FLAG epitope was tagged to the N-terminal end of IKK-i gene. Restriction enzyme SalI sites were constructed in the 5'- and 3'-ends of FLAG-hIKK-i fragment using primer sequences (1) and (2) of the following formulae by applying with PCR.

(1)5'-gggtcgacca ccatggacta caaggacgac gatgacaaga tgcagagcac agccaat-3'

(2)5'-gtcgactcag accatcagga ggtgc-3'

The resulted sequence was subcloned into T-vector (p-GEM-T)(Promega), excised by using the restriction enzyme SalI, and subcloned into pEF-BOS expression vector to construct the expression vector pEF-BOS-FLAG-WT-IKK-i.

Example 10 (Activation of NF-κB by IKK-i)

The NF-κB consensus reporter construct (pNF-κB-Luc)(Stratagene Inc.) and IKK-i expression vector, 0 μg (without addition), 0.3μg, 1.0μg and 3.0μg of pEF-BOS-FLAG-WT-IKK-i obtained in example 9 were added respectively to 3×10^5 cells of 293T cells, and transiently cotransfected by lipofection using Tran it LT-1 (Pan Vera Corp.). Then luciferase activity was assayed by using Dual Luciferase Reporter assay system (Promega Inc.). Vector only was added for the control group.

The total amount of DNA was regularized to 4 μg by using pEF-BOS vector.

Results are shown in Fig. 9. The lower part of Fig. 9 shows the amount of protein, which was determined by immunoblotting using anti-FLAG antibody (M2).

Example 11 (Preparation of a mutant of IKK-i, in which No. 38 lysine is replaced by alanine)

The bases coding No. 38 lysine in IKK-i were replaced by the bases coding alanine and a gene of IKK-i mutant was prepared by means of point mutation technique. Transformer site directed mutagenesis kit (Clontech Inc.) was used for point mutation.

Example 12 (Construction of expression vector of mutant of IKK-i)

The expression vector of the mutant, pEF-BOS-FLAG-K38A-IKK-i, was prepared by the same way as of in example 9 using the mutant gene obtained in example 11.

Example 13 [Preparation of GST-IκB-αN protein (WT)]

A gene coding amino acids 1 - 72 in the amino acid sequence, in which the C-terminal part was split from ankyrin repeat in IκB-α, was prepared and this was expressed in E. coli. The resulted product was purified by using glutathione Sepharose to obtain GST-IκB-αN protein (WT). PGEX2T (Pharmacia Inc.), was used as the vector.

Example 14 [Preparation of GST-IκB-αN protein (AA)]

A gene, in which both of bases coding for the No. 32 and No. 36 serine

residues of GST-IκB-αN were replaced by the bases coding for alanine, was prepared. This was expressed in E. coli and purified using glutathione Sepharose to obtain GST-IκB-αN protein (AA).

Example 15 (Phosphorylation of serine residue of IκB-α by IKK-i)

The pEF-BOS-MOCK, the pEF-BOS-FLAG-WT-IKK-i obtained in example 9, or the mutant construct (pEF-BOS-FLAG-K38A-IKK-i) obtained in example 12, was transiently transfected to 2×10^6 cells of 293T-cells on 10 cm dishes. After 24 hours, expressed IKK-i protein or K-38A-IKK-i protein was purified by means of immunoprecipitation and measured by in vitro kinase assay.

The GST-IκB-αN protein (WT) obtained in example 14 or the protein (AA) obtained in example 15 was used as a substrate for the in vitro kinase assay.

IKK-i or K38A-IKK-i, substrates and [γ-^{32}P]ATP were reacted at 30℃ for 20 minutes. The reaction mixture was developed with SDS-PAGE and measured by autoradiography.

The results are shown in Fig. 10. In Fig. 10, the arrow pointing to a position of about 80 kDa shows a band of autophosphorylation and the arrow below it shows a band of GST-IκB-αN. The molecular weight (kDa) is shown on the left side in Fig. 10. The lower part of Fig. 10 shows the results of determination of the amount of protein by immunoblotting using anti-FLAG antibody (M2).

As a results, K38A-IKK-i did not phosphorylate GST-IκB-αN protein (WT) though IKK-i phosphorylated GST-IκB-αN protein (WT). Further, IKK-i did not phosphorylate GST-IκB-αN protein (AA).

Example 16 (Isolation of the molecule which interacts with IKK-i using yeast two

hybridization)

Amino acids No. 541 - 716 from human IKK-i were inserted into a plasmid, pAS2-1, for expression of a chimera protein with a GAL4 DNA binding domain in order to prepare a bait plasmid. This was then transformed to yeast Y190 and grown on a selective medium. The transformant thus obtained was further transformed with a pACT2 plasmid containing a human B-cell derived cDNA library, which was able to express the chimera protein with a GAL4 activation domain. The cells were then grown on a selective medium. The plasmid was recovered from the positive clones thus obtained. Finally the base sequence was determined by using a DNA sequencer. As a result of a homology search, 10 clones were found to be identical to the sequence I-TRAF/TANK which had been already known.

Example 17 (Binding with IKK-i and I-TRAF in cells)

Flag was linked to the N-terminal of human IKK-i as an epitope, and then inserted into the expression vector pEF-BOS.

Myc was added to the N-terminal of human I-TRAF as an epitope, and then inserted into the expression vector pEF-BOS.

Further, the Flag was linked to the N-terminal of human IKK-i as an epitope, and a sequence, in which Myc was added to the N-terminal of human I-TRAF as an epitope, was added thereto, then inserted into the expression vector pEF-BOS.

These were transfected into a monkey kidney cell line, COS-7 cells, by lipofection. After 24 hours, the cells were solubilized with buffer containing 1.0% Nonidet P-40. The resulting solubilized material was immunoprecipitated by anti-Flag antibody or anti-Myc antibody, and was then analyzed by means of western blotting using anti-Myc antibody or anti-Flag antibody.

Results are shown in Fig. 11. In Fig. 11, lane 1 shows Flag-IKK-I, lane 2 shows Myc-I-TRAF, lane 3 shows Flag-IKK-i and Myc-I-TRAF respectively.

Example 18 (Determination of binding region of I-TRAF and IKK-i)

First, three types of I-TRAF defective mutants consisting of a fragment of the amino acid sequence, amino acids No. 1 - 170, 1 - 247 or 193 - C-terminal (stop) of human I-TRAF, to which Myc was added to the N-terminal thereof, were prepared. These were inserted into pEF-BOS respectively, then transfected with Flag-IKK-i into COS-7 cells. The cells were solubilized after 24 hours, and the resulting solubilized materials were immunoprecipitated with anti-Myc antibody, then analyzed by means of western blotting with anti-Flag antibody.

The results are shown in Fig. 12. In Fig. 12, lane 1 shows 1 - 170 fragment, lane 2 shows 1 - 247 fragment, lane 3 shows 193 - stop fragment and lane 4 shows FL (full length) respectively.

Example 19 (Confirmation of phosphorylation of I-TRAF by IKK-i)

Flag-IKK-i and Myc-I-TRAF were transfected into COS-7 cells and solubilized after 24 hours. After immunoprecipitating the resulting solubilized material with anti-Flag antibody or anti-Myc antibody, kinase buffer and [γ-^{32}P]ATP were added to the precipitate and allowed to react. Then an in vitro kinase assay was performed.

The results are shown in Fig. 13. Lanes 1, 3 and 5 show the results of transfection with Flag-IKK-I only, and lanes 2, 4 and 6 show the results of transfection with both Flag-IKK-i and Myc-I-TRAF. As shown in Fig. 13, bands of phosphorylated Myc-I-TRAF were detected in evey immunoprecipitates with anti-

Flag antibody (lane 2) or anti-Myc antibody (lane 4). Consequently, it was clearly demonstrated that Myc-I-TRAF was a substrate which was phosphorylated by IKK-i. The amounts of expression of protein in each lane were confirmed by means of western blotting analysis of the products of solubilizing with anti-Flag antibody (upper part of lanes 5 and 6) or anti-Myc antibody (lower part of lanes 5 and 6).

Example 20 (Determination of phosphorylated region in I-TRAF)

Myc-I-TRAF defective mutants [1 - 170, 1 - 247, 197 - stop and full length (FL)] were transfected with IKK-i into COS-7 cells and the resulting solubilized material was obtained. After that, immunoprecipitation was performed with anti-Flag antibody or anti-Myc antibody and an in vitro assay was performed.

The results are shown in Fig. 14. In Fig. 14, lanes 1 and 5 show a fragment of amino acids No. 1 – 170, lanes 2 and 6 show a fragment of amino acids No. 1 – 247, lanes 3 and 7 show a fragment of amino acids No. 197 - stop and lanes 4 and 8 show full length (FL) respectively.

Example 21 (Phosphorylation of I-TRAF protein by IKK-i)

Human I-TRAF cDNA was then inserted into an expression vector pGEX-5X-i, which was able to be expressed as a chimera protein with glutathione S transferase (GST) in E. coli. The vector thus obtained was transformed into E. coli DH5α. After E. coli was cultured overnight in LB liquid medium, IPTG was added and the mixture was incubated for 3 hours. The collected E. coli cells were solubilized by PBS and disrupted by ultrasonication. Triton X-100 was added up to 1% to solubilize the mixture, glutathione Sepharose was added and the mixture was allowed to react for 1 hour. GST-I-TRAF protein bound with glutathione Sepharose

was eluted with a glutathione solution and used as the purified protein for the experiment.

Next, Flag-IKK-i was transfected into COS-7 cells. Simultaneously, a mutant IKK-i (K38A) was prepared and transfected.

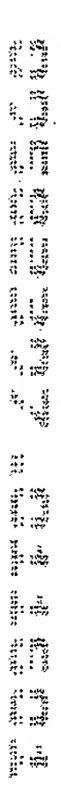
After 24 hours of transfection, the cells were solubilized and immunoprecipitated with anti-Flag antibody. A purified GST-I-TRAF (1.0 μg) was added to the immunoprecipitate and phosphorylated in vitro to perform a kinase assay.

The results are shown in Fig. 15. In the cells in which Flag-IKK-i was expressed, phosphorylation of GST-I-TRAF was observed (Fig. 15, upper part of lane 1). No phosphorylation was observed with the K38A mutant (Fig. 15, upper part of lane 2). Further, intracellular expression of the K38A was confirmed by means of western blotting analysis of the product of solubilizing with anti-Flag antibody (Fig. 15, lower part).

Industrial Applicability

The present invention provides a novel IκB kinase, IKK-i, which is a novel serine/threonin kinase capable of activating transcription factor NF-κB which regulates expression of various genes involved in immune response. The present invention also provides the gene coding for the same and a pharmaceutical composition containing the same. Since IKK-i of the present invention phosphorylates IκB and activates NF-κB, it is effective for improvement of the immune response function and for treatments for inflammatory diseases to regulate this gene. Further, IKK-i of the present invention binds with I-TRAF to phosphorylate the same, and is effective for regulation of TRAF molecule activity

involved in apoptosis.



CLAIMS:

1. A protein comprising an amino acid sequence represented by SEQ ID NO: 2 or SEQ ID NO: 4, or an amino acid sequence, in which one or more amino acids in the said amino acid sequence are deleted or replaced by other amino acids, and/or one or more other amino acids are added, and capable of activating transcription factor NF-κB.

2. The protein according to claim 1 which is serine/threonine kinase.

3. The protein according to claims 1 or 3 which has activities for binding with I-TRAF and for phosphorylating I-TRAF.

4. A gene comprising base sequence coding the protein according to claim 1.

5. The gene according to claim 4, wherein the base sequence comprises the base sequence represented by SEQ ID NO: 1 or SEQ ID NO: 3.

6. A pharmaceutical composition comprising the protein according to any one of claims 1 - 3 and a pharmaceutically acceptable carrier.

7. The pharmaceutical composition according to claim 6, which acts on the immune response mechanism.

8. The pharmaceutical composition according to claim 6, which is a preventive or therapeutic agent for diseases involving the I-TRAF or the TRAF molecule.

ABSTRACT

Novel IkB kinase IKK-i which is a novel serine/threonine kinase capable of activating a transcription factor NF-kB which inhibits the expression of various genes relating to immune response; a gene encoding the same; and medicinal compositions containg the same.

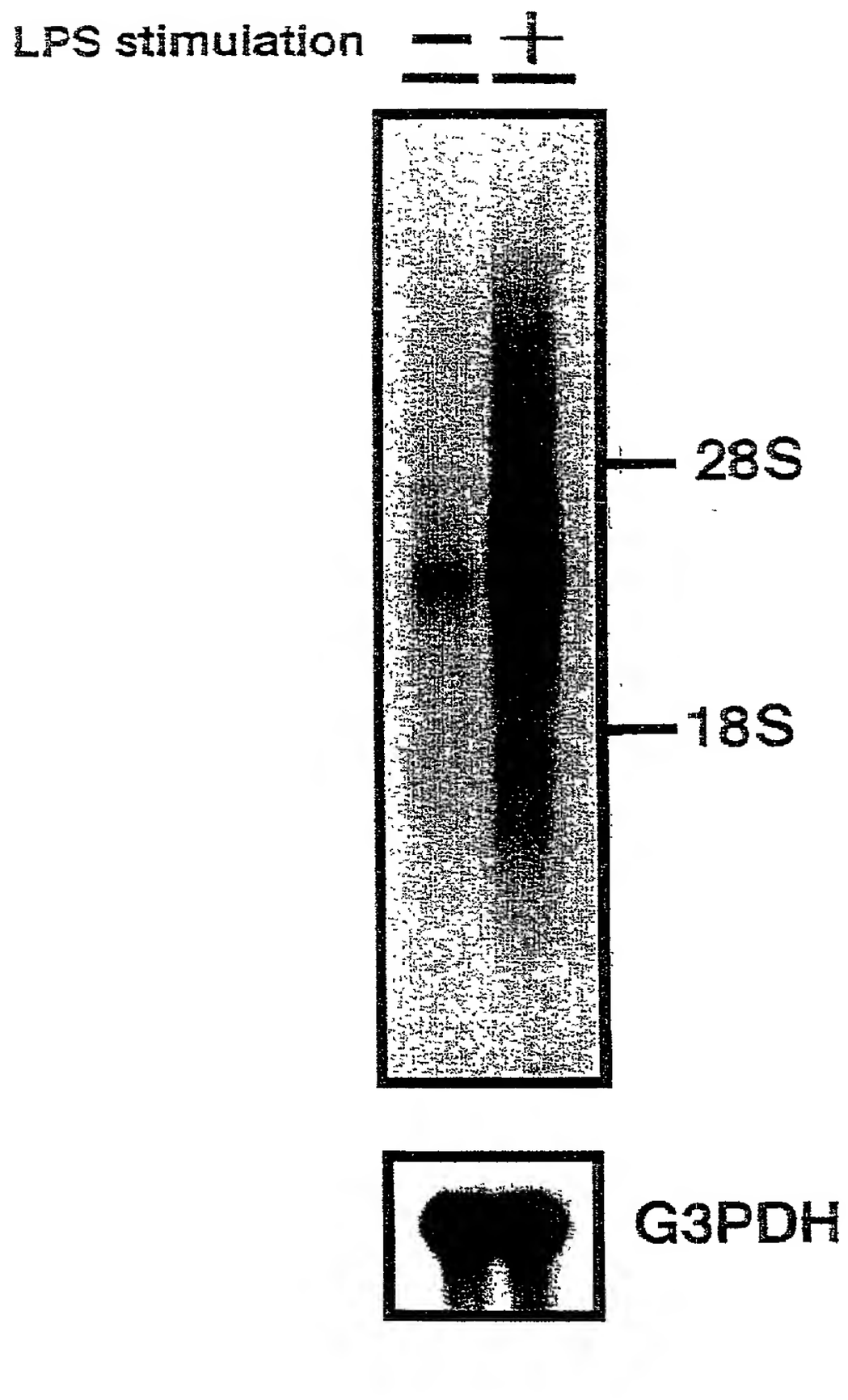


Figure 1

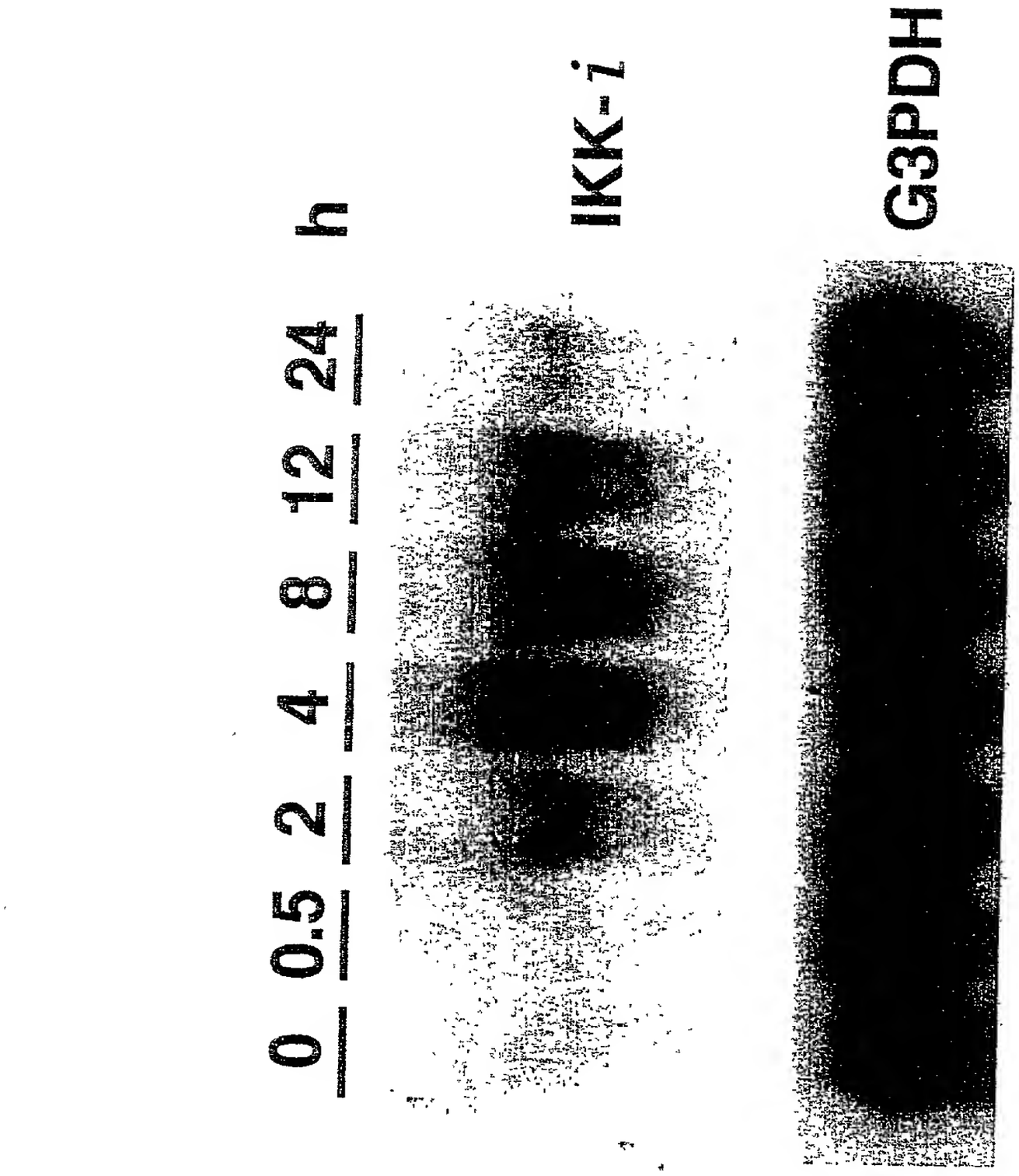


Figure 2

```
hIKK-i    1:MQSTANYIWHTDDLLGQGATASVYKARNKKSGELVAVKVFNTTSYLRPRELQVREFEVLRKLNHQNIVKLFAVEE  75
mIKK-i    1:MQSTTNYIWHTDDLLGQGATASVYKARNKKSGEVVAVKVFNSASYRRPFEVQVREFEVLRRLNHQNIVKLFAVEE  75

hIKK-i   76:TGGSRQKVLVMEYCSSGSLLSVLESPENAFGLPEDEFLVVLRCVVAGMNHLRENGIVHRDIKPGNIMRLVGEEGQ 150
mIKK-i   76:TGGSRQKVLIMEYCSSGSLLSVLEDPENTFGLSEEEFLVVLRCVVAGMNHLRENGIVHRDIKPGNIMRLVGEEGQ 150

hIKK-i  151:SIYKLTDFGAARELDDDEKFVSVYGTEEYLHPDMYERAVLRKPQQKAFGVTVDLWSIGVTLYHAATGSLPFIPFG 225
mIKK-i  151:SIYKLSDFGAARKLDDDEKFVSVYGTEEYLHPDMYERAVLRKPQQKAFGVTVDLWSIGVTLYHAATGSLPFIPFG 225

hIKK-i  226:GPRRNKEIMYRITTEKPAGAIAGAQRRENGPLEWSYTLPITCQLSLGLQSQLVPILANILEVEQAKCWGFDQFFA 300
mIKK-i  226:GPRRNKEIMYRITTEKPAGAISGTQKQENGPLEWSYSLPITCRLSMGLQNQLVPILANILEVEEDKCWGFDQFFA 300

hIKK-i  301:ETSDILQRVVVHVFSLSQAVLHHIYIHAHNTIAIFQEAVHKQTSVAPRHQEYLFEGHLCVLEPSVSAQHIAHTTA 375
mIKK-i  301:ETSDILQRTVIHVFSLPQAVLHHVYIHAHNTIAIFLEAVYEQTNVTPKHQEYLFEGHPCVLEPSLSAQHIAHTAA 375

hIKK-i  376:SSPLTLFS-TA-IPKGLAFRDPALDVPKFVPKVDLQADYNTAKGVLGAGYQALRLARALLDGQEELMFRGILHWVME 448
mIKK-i  376:SSPLTLFSMSSDTPKGLAFRDPALDVPKFVPKVDLQADYSTAKGVLGAGYQALWLARVLLDGQALMLRGLHWVLE 450
                                  *       *       *       *       *

hIKK-i  449:VLQATCRRTLEVARTSLLYLSSSLGTERFSSVAGTPEIQELKAAAELRSRLRTLAEVLSRCSQNITETQESLSSL 523
mIKK-i  451:VLQDTCQQTLEVTRTALLYLGSSLGTERFSSGSGMPDVQERKEATELRTRLQTLSEILSKCSHNVTETQRSLSCL 525
                                  *               *       *       *       *

hIKK-i  524:NRELVKSRDQVHED-RSIQQIQCCLDKMNFIYKQFKKSRMRPGLGYNEEQIHKLDKVNFSHLAKRLLQVFQEECV 597
mIKK-i  526:GEELLKNRDQIHEDNKSIQKIQCCLDKMHFIYKQFKKSRMRPGLSYNEEQIHKLDKVNFSHLAKRLLQVFQEECV 600
              *       *       *       *

hIKK-i  598:QKYQASLVTHGKRMRVVHETRNHLRLVGCSVAACNTEAQGVQESLSKLLEELSHQLLQDRAKGAQASPPPIAPYP 672
mIKK-i  601:QTYQVSLVTHGKRMRQVQRAQNHLHLIGHSVATCNSEARGAQESLNKIFDQL--LLDRASEQGAEVSPQPMAPHP 673

hIKK-i  673:SPTRKDLLLHMQELCEGMKLLASDLLDNNRIIERLNRVPAPPDV                                716
mIKK-i  674:GPDPKDLVFHMQELCNDMKLLAFDLQDNNRLIERLHRVPSAPDV                                717
```

Figure 3

```
hIKK-i    1 MQSTANYL------WHTDDLIGQGATASVYKARNKKSGELVAVKVFNTTSYLRPRDVQVREFEVLKKLNHQNIVKLFAVEET----GGSRQKVIVMEYCS  90
hIKK-β    1 MSWSPSLRTQTCGAWEMKERLGTGGFGNVIRWHNQETGEQIAIKQCRQELSPRNRERWCLEIQIMRRLTHPNVVAARDVPEGMQNLAPNDLPLLAMEYCQ 100
hIKK-α    1 MERPPGLRPGAGGPWEMRERLGTGGFGNVCLYQHRELDLKIAIKSCRLELSTKNRERWCHEIQIMKKLNHANVVKACDVPEELNILI-HDVPLLAMEYCS  99

hIKK-i   91 SGSLLSVLESSEKNAFGLPEDEFLVVLRCVVAGMNHLRENGIVHRDIKPGNIMRLVGEEGQSLYKLTDFGAARELEDDEKFVSVYGTEEYLHPDMYERAVL 190
hIKK-β  101 GGDLRKYLNQFENCCGLREGAILTLLSDIASALRYLHENRIIHRDLKPENIV-LQQGEQRLIHKTIDLGYAKELDQGSLCTSFVGTLQYLAPELLE---- 195
hIKK-α  100 GGDLRKLLNKPENCCGLKESQILSLLSDIGSEIRYLHENKIIHRDLKPENIV-LQDVGGKIIHKIIDLGYAKDVDQGSLCTSFVGTLQYLAPELFE---- 194

hIKK-i  191 RKPQDKAFGVDTVDLWSIGVTLYHAATGSLPFIE------FGGPRRNKEIMYRITTDKPAGAIA-GAQRRENGPIEWSYTLEITCQLSFGLQSQLVPILAN 283
hIKK-β  196 ----DQKYEVDYVWSEGTLAEFCITCVRPDLKPNWDPVQWHSKVRQKSEVDIVVSEDLNGTVKFSSSLPYPNNLNSVLAERLEKWLQLMLMWHPRQRGT- 290
hIKK-α  195 ----NKPYTATVDYWSFGTMVFECIAGYRPFLHHLQPFTWHEKIKKKDPKCIFACEEMSGEVRFSSHLPQPNSICSLIVEPMENWLQLMLNWDPQQRGGP 290

hIKK-i  284 I-LEVEQAKCWGFDQFFAETSDILQRVVVHVFSLSQAVLHHIYIHAHNTIAIFQEAVHKQLSVAPRHQEYLFFGHLFCVLEPSVSAQHTAHTTASSPLT-- 380
hIKK-β  291 -DPLYGPNGC------FKALDDILNLKLVHILNMVTGTLETIPYPVTEESISQSLKARTQQDLGIEPEEDQELLQEAGLALIPDKPATQCISDGKLNEGHTLD 383
hIKK-α  291 VDLTLKQPRG------FVLMDHTLNNLKIVHTLNMTSAKIISFLLPPDESLHSLQSRLEREIGFNTGSQEFLSETGISLDPRKPASQCV----LDGVRGCD 380

hIKK-i  381 -----LFS---LAIPKGLAFRDPALDVPKFVPKVDLQADYNTAKGVLGAGYQALRLARA----LLDGQELMFRGEHWV---MEVLQATCRRTLEVARTSL 465
hIKK-β  384 MDLVFLFDNSKKITVETQISPRPQPESVSCILQEPKRNLAFFQLRKVWGQVWHSIQTLKEDCNRLQQGQRAAMMNLLRNNSCLSKMKNSMASMSQQLKAKL 483
hIKK-α  381 SYMVYLEDKSKQVVEGPFASRSLSDCVNYLVQDSKIQLPIIQLRKVWAEAVHYVSGLKEDYSRLFQGQRAAMLSLLRYNANLTKMKNTLISASQQLKAKL 480

hIKK-i  466 LYLSSS--LGTERFSSVAGTPEIQELKAAAEEVSRLRTLAIVLSRQSQN--ETETQFSLSSINRELVKSRDQVHEDRSIQQIQCCLDKMNFIVKQFK-KS 560
hIKK-β  484 DFFKTSIQIDTFKYSEQTEF----GETSDKLLLAW-REMEQAVELQGRENEVKLIVERMMAIQTDTVDLQRSPMGRKQGGTLDDLEEQARELYRRLREKP 578
hIKK-α  481 EFEHKSTQLDLERYSEQMTY----GISSEKMLKAW-KEMELKAIHYAEVGVIGYLEDQIMSLHAETMELQKSPYGRQQGDLMESLEQRAIDLYKQLKHRP 575

hIKK-i  592 RMRPGLGYNELQIH----------KLDKVNESHIAN------RLLQVFQEECVQKYQASLVTHGKRMRVVHETRN----HIRLVGGSVAACNTEAQGVQE 640
hIKK-β  579 RDQRTEGDSQEMVRLLLQAIQSFEKKVRVIYTQLSKTVVCKQKALELLPKK--VEEVVSLMNEDEKTVVRLQEKRQKELWNLLKIACSK---------VRG 667
hIKK-α  576 SDH-SYSDSTEMVKIIVHTVQSQDRVLKELSGHMFSKLLGQKQKIIDLLPK--VEVALSNIKEADNTVMFMQGKRQKEIWHLLKIAGTQSSARSL---VGS 669

hIKK-i  641 SLSKLLEELSHQLTQDRAKGAQASPPPIAPYESETRK--DLLLHMQELCEGM--KLLASDLLDNNRIIERLNRVPAPPD----------V         716
hIKK-β  668 PVSGSPDSMNASRLSQPGQLMSQPSTASNSLIELAKKSEELVAEAHNLQ-TLLENATQDTVREQDQSFTALDWSWLQTEEEEHSCLEQAS         756
hIKK-α  670 SLEGAVTPQTSAWLP-RTSAEHDHSLSCVVTPQDGETSAQMIEENLN-CLGHLSTIDHEANELQGNSMMNLDWSWL----------TE         745
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Figure 4

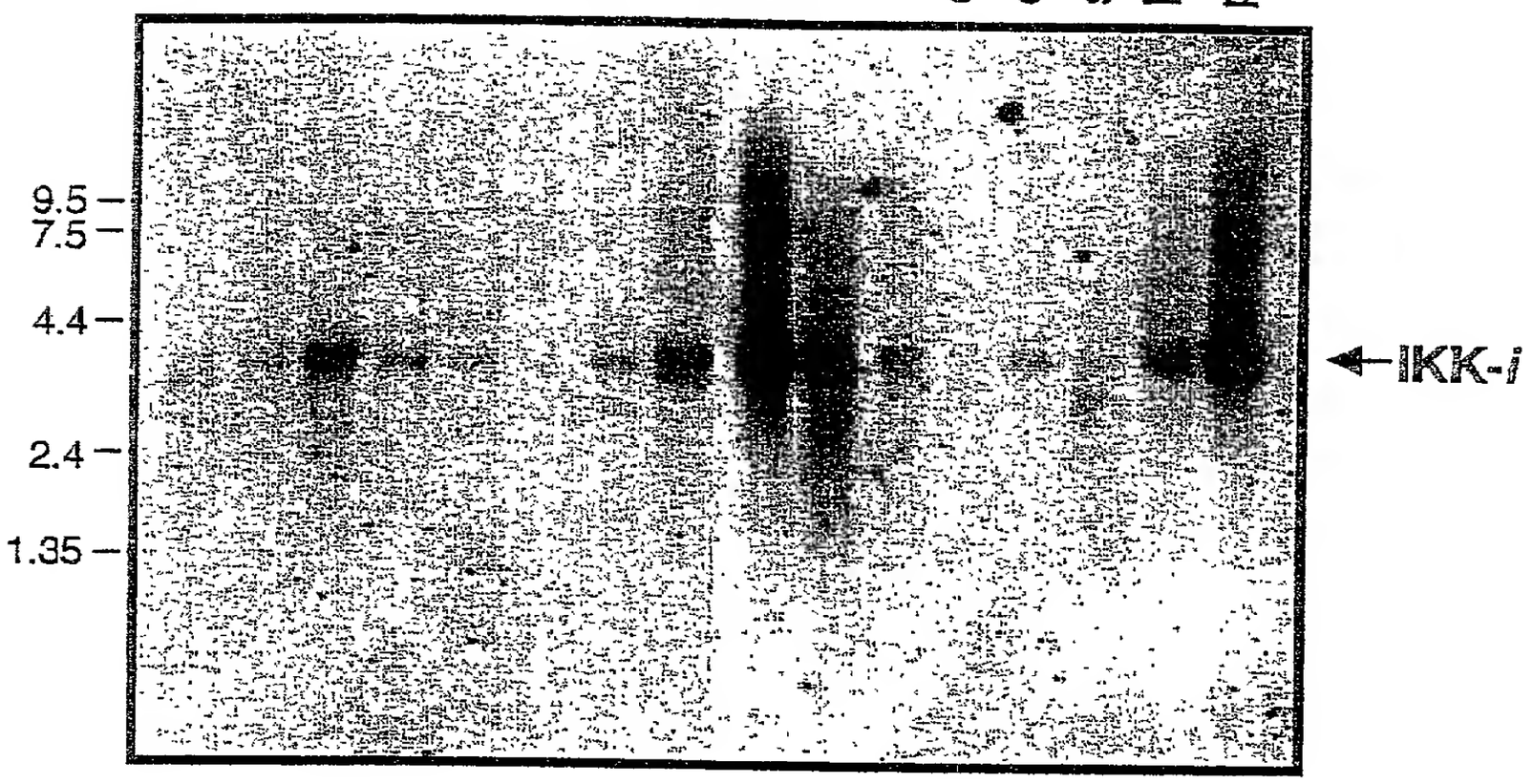

heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
thymus
prostate
orchis
ovarium
small intestine
large intestine
peripheral leukocyte
9.5
7.5
4.4
2.4
1.35
IKK-i

Figure 5

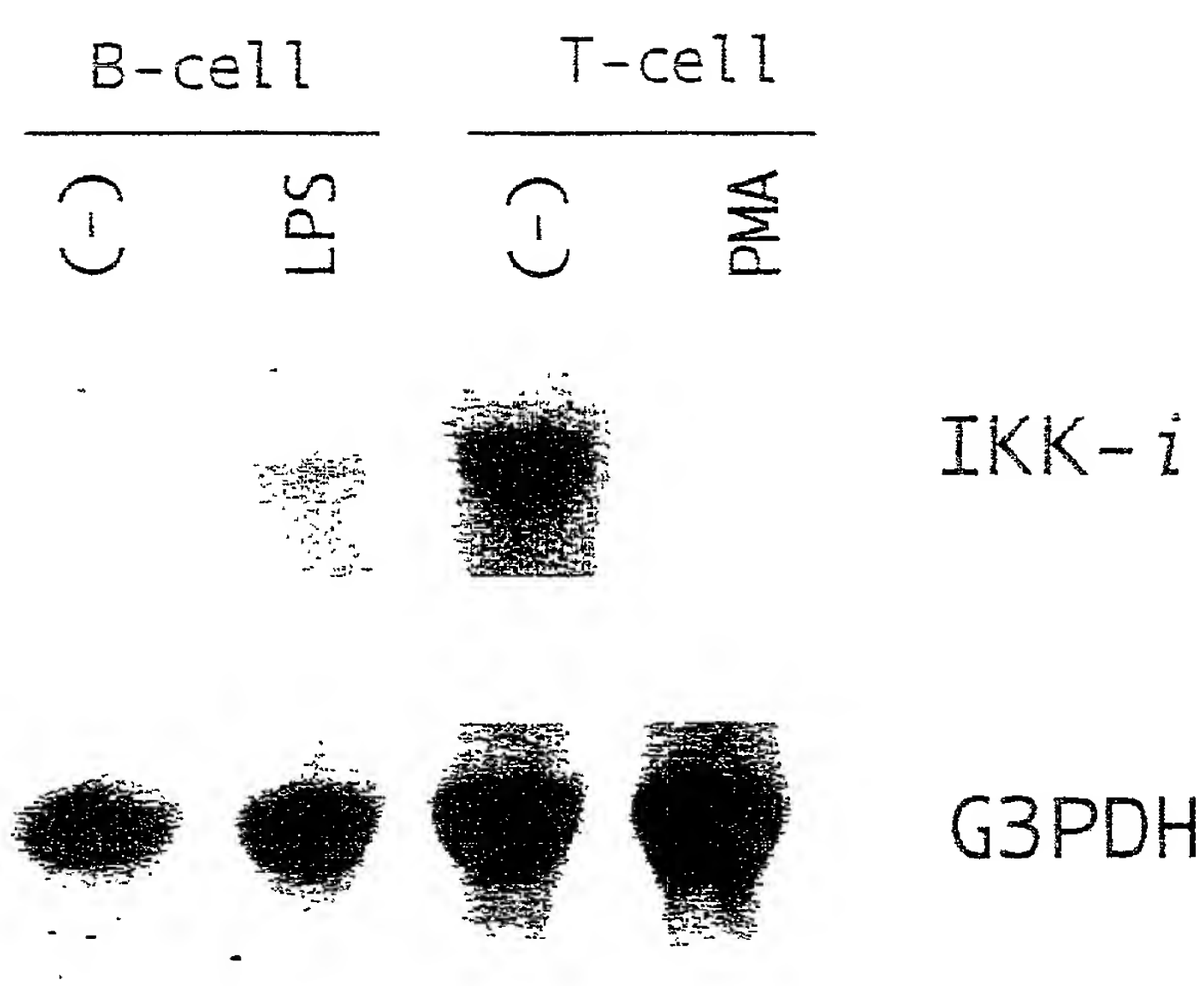


Figure 6

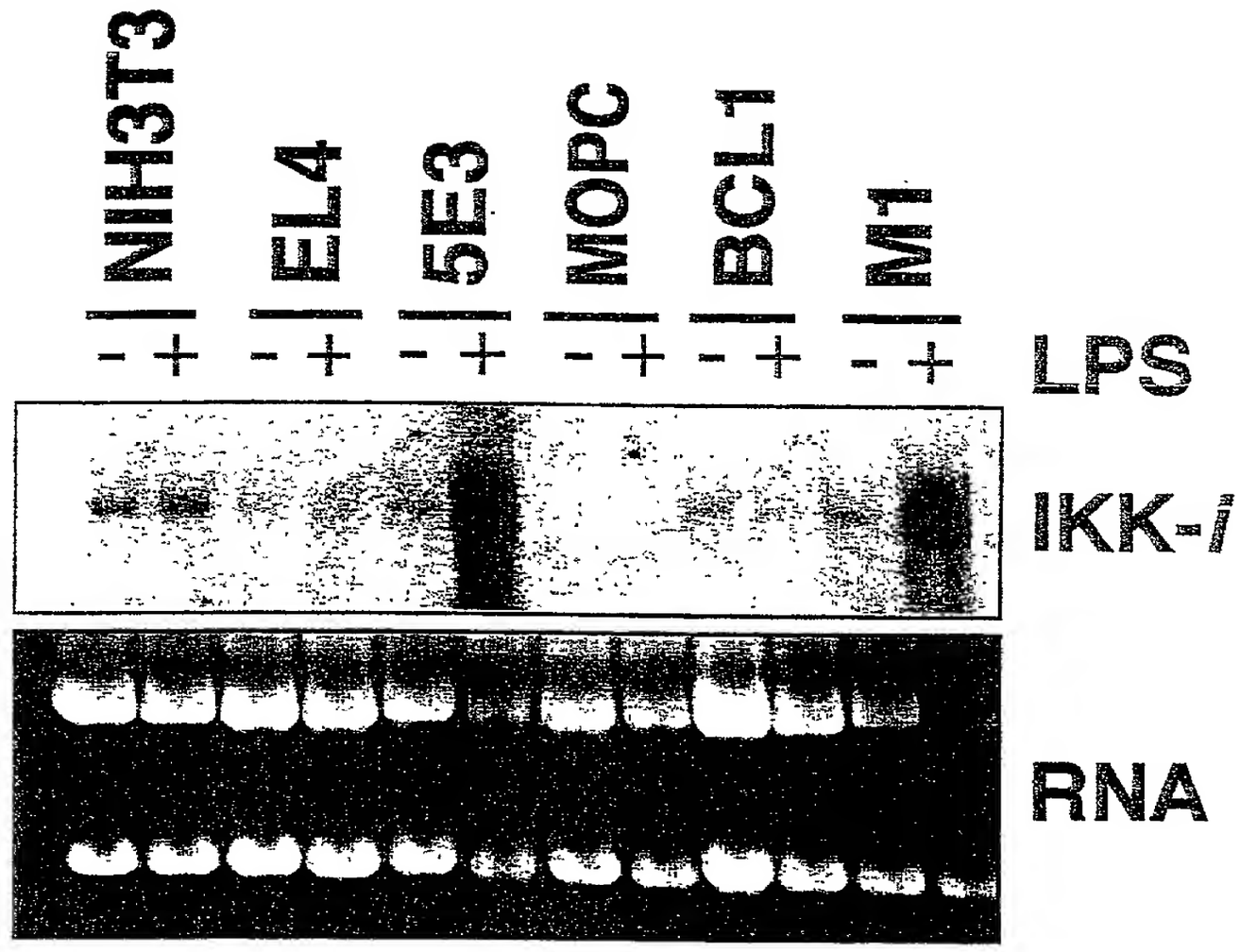


Figure 7

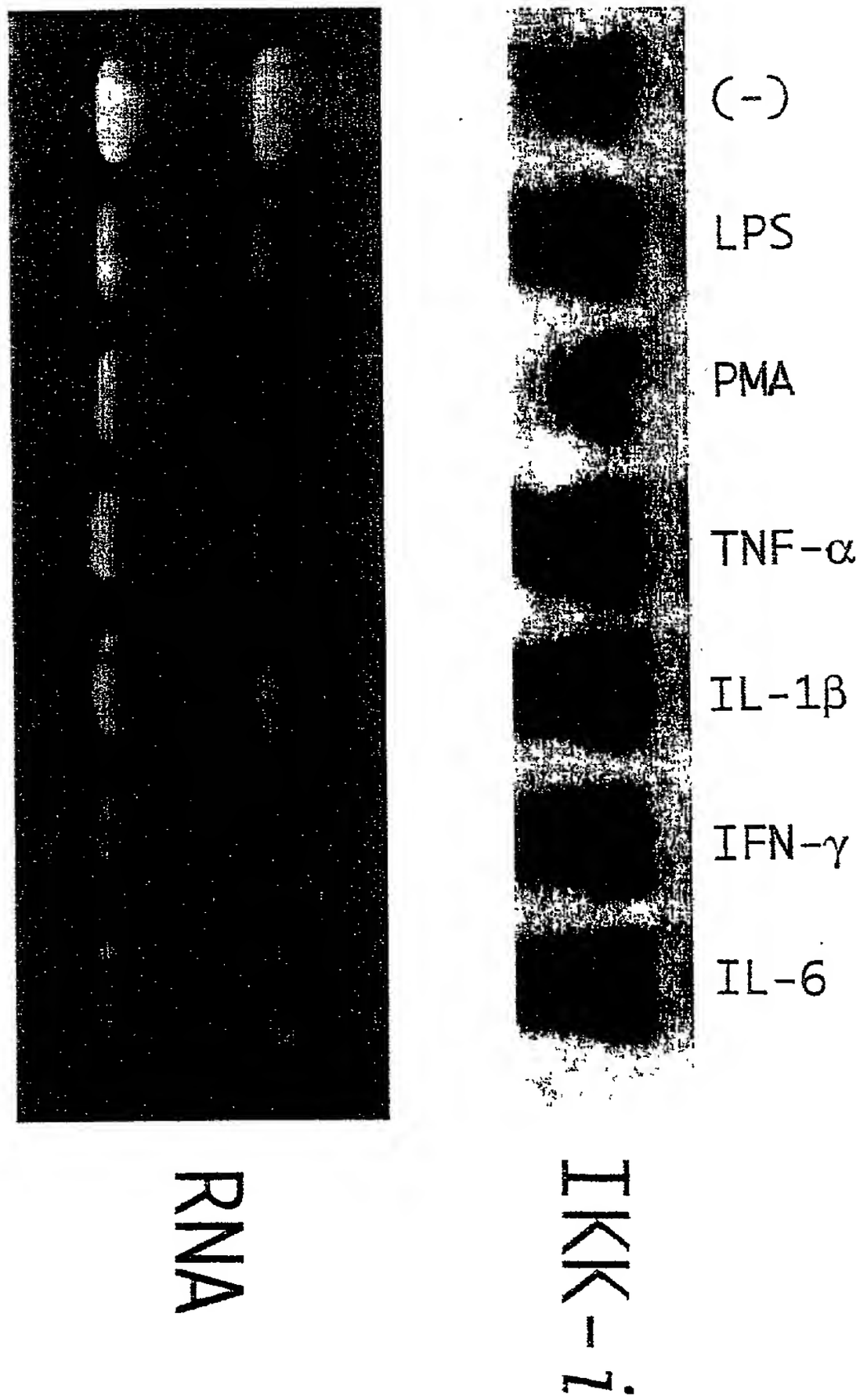
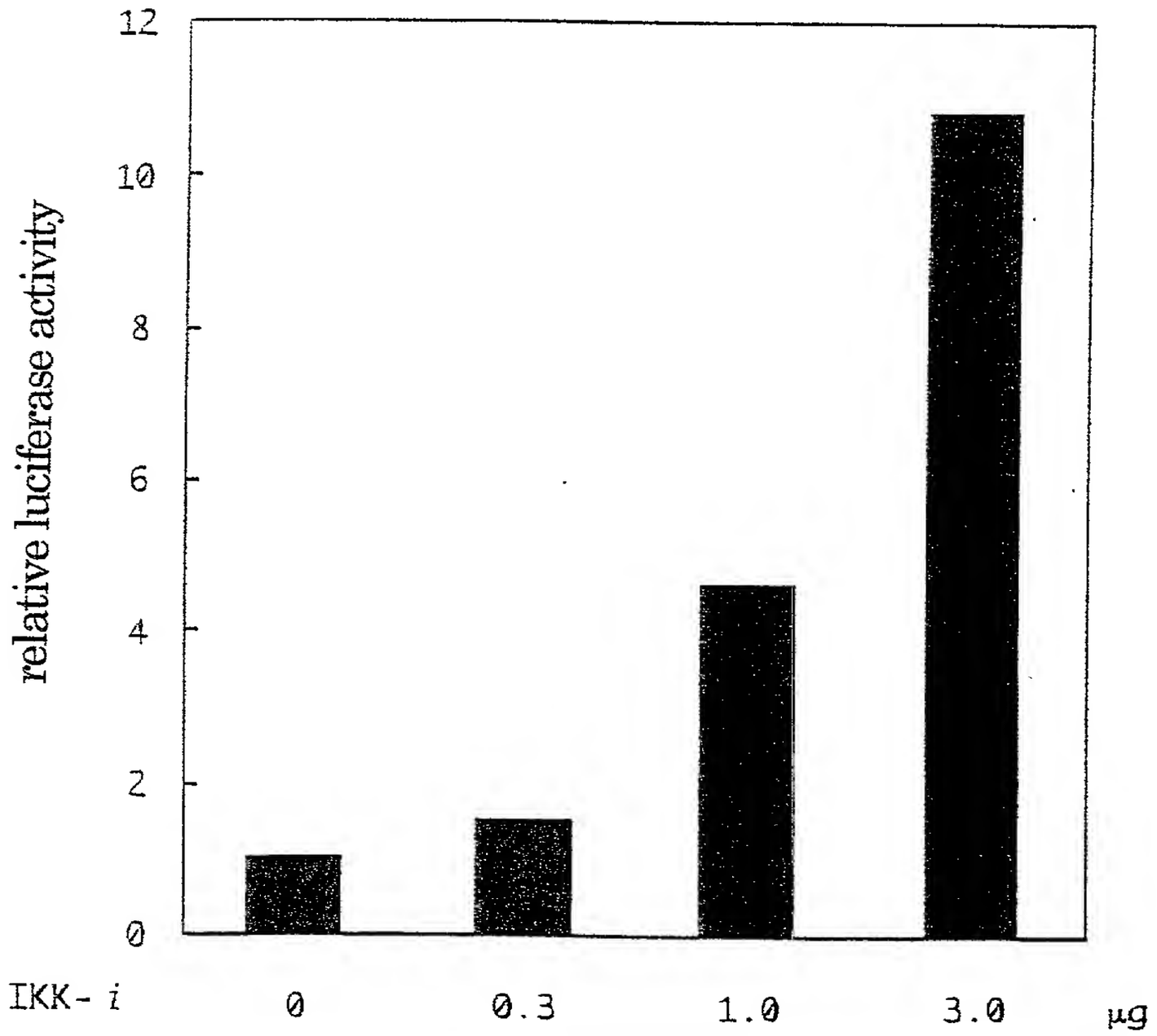


Figure 8

over expression of IKK- i activates NF-κB



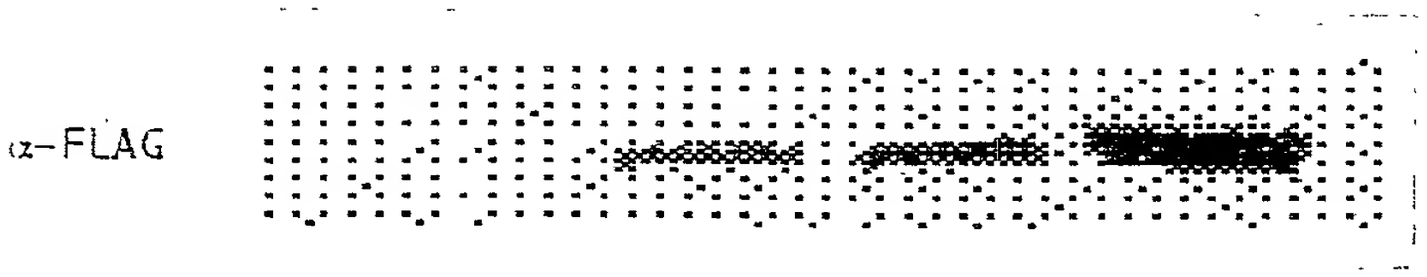


Figure 9

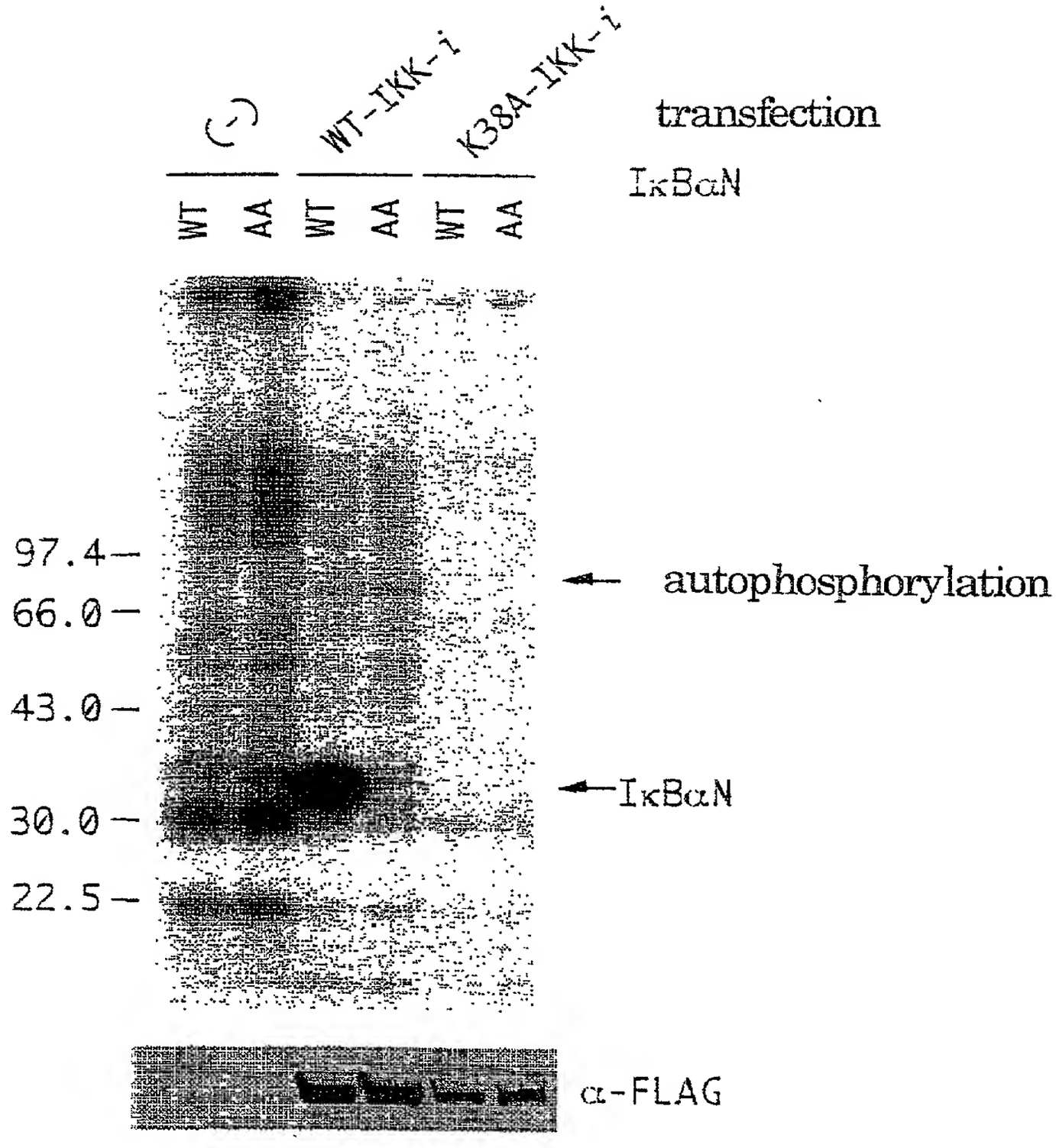

(-)
WT-IKK-i
K38A-IKK-i
transfection
WT
AA
WT
AA
WT
AA
IκBαN
97.4
66.0
43.0
30.0
22.5
autophosphorylation
IκBαN
α-FLAG

Figure 10

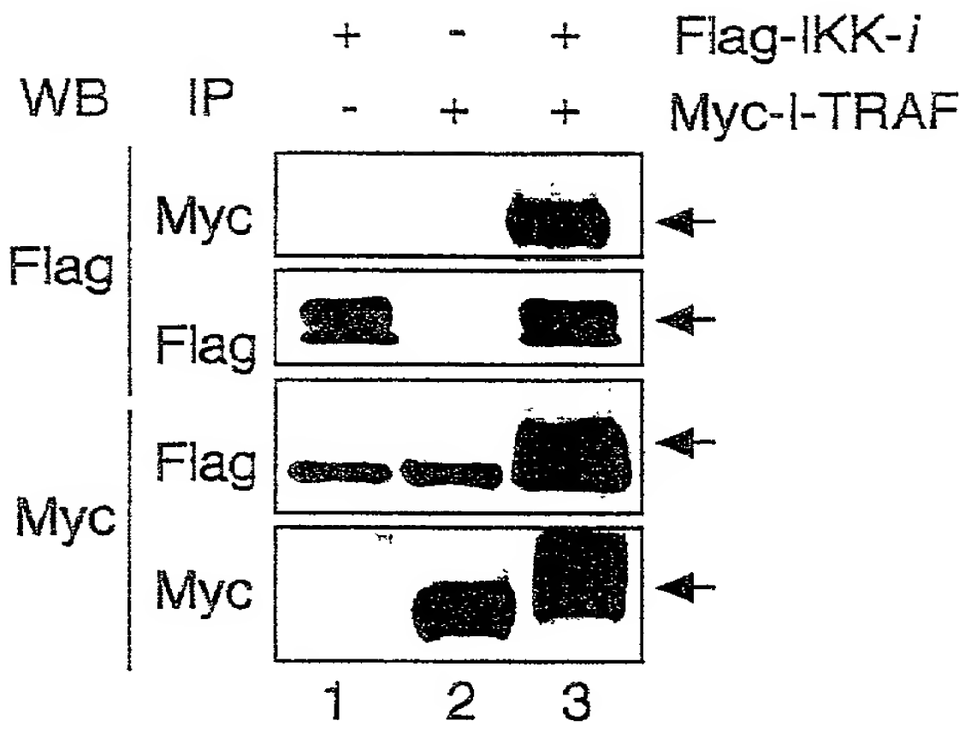


Figure 11

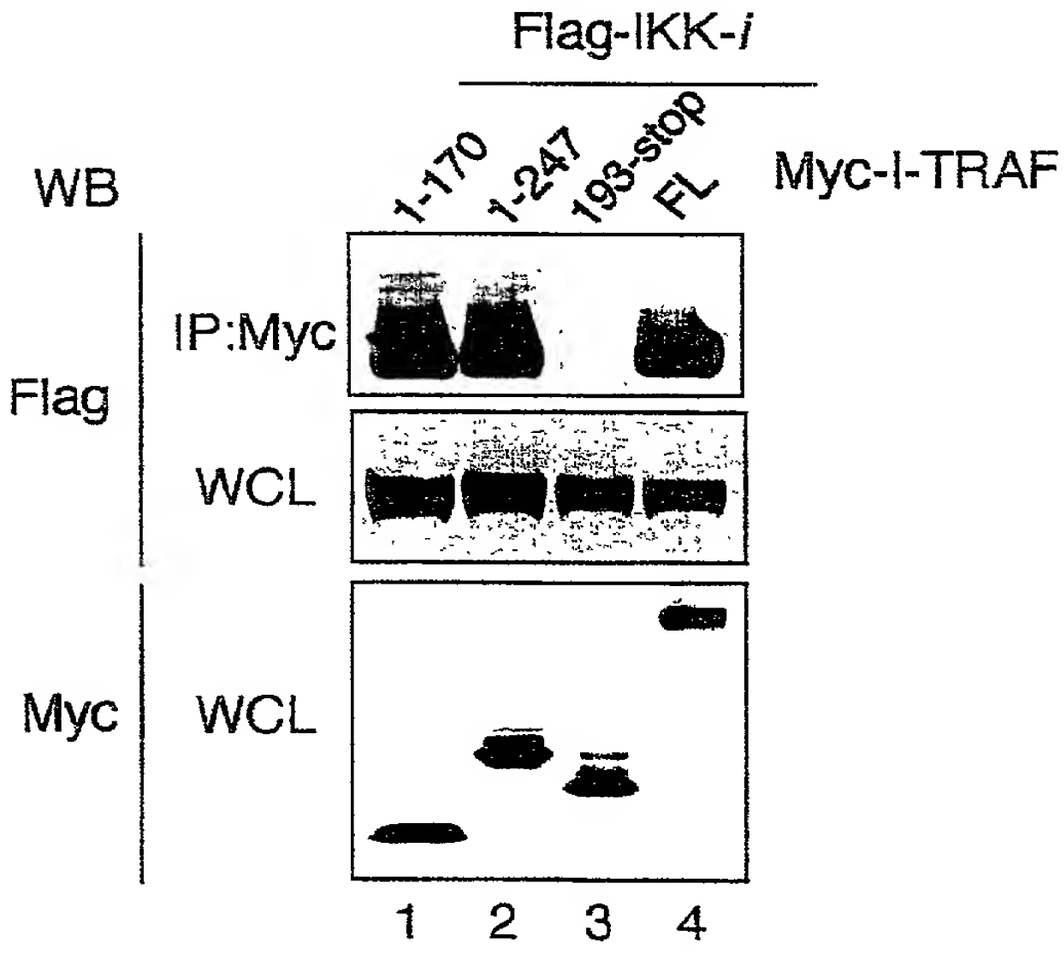


Figure 12

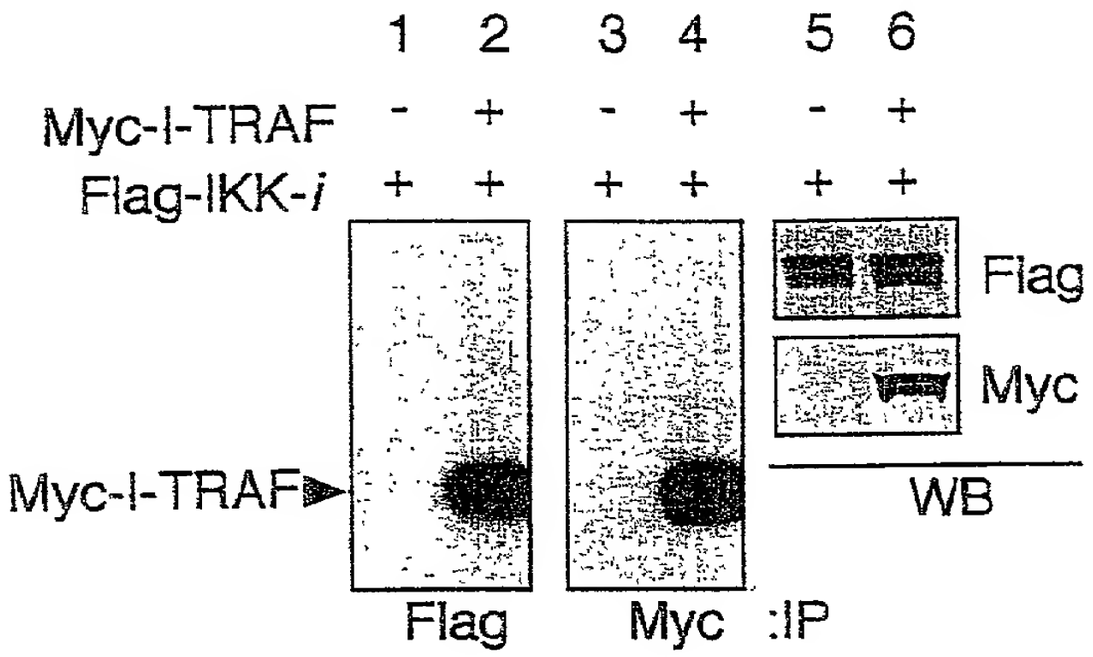


Figure 13

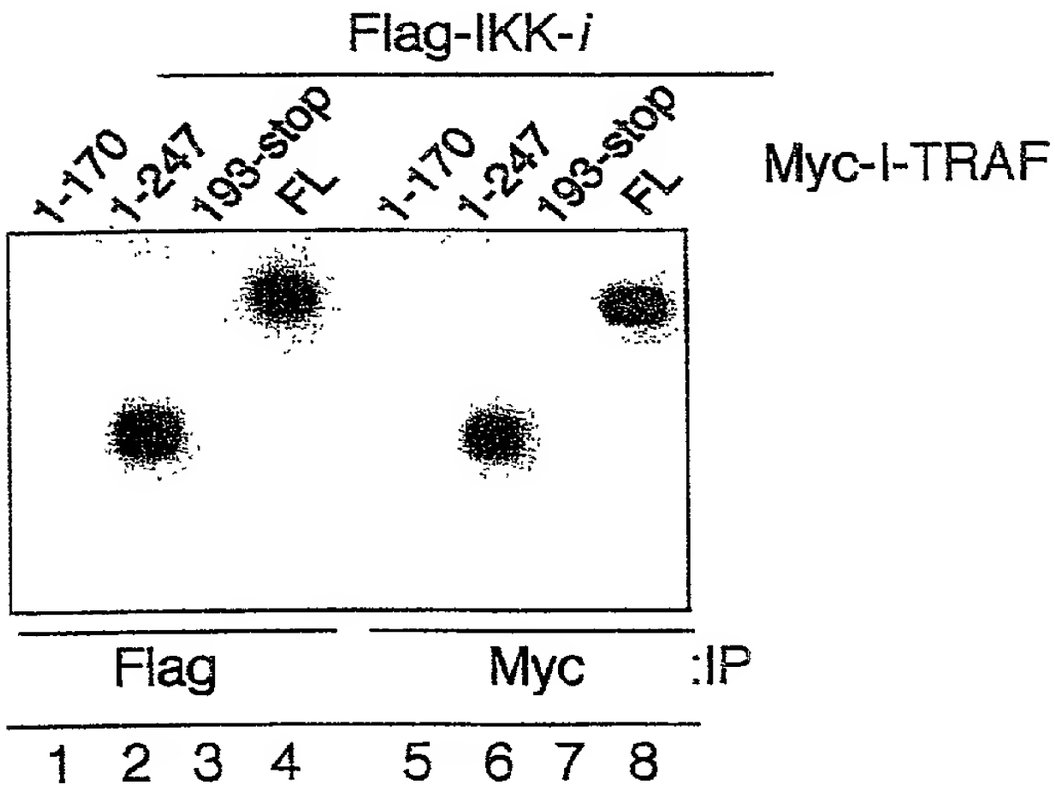


Figure 14

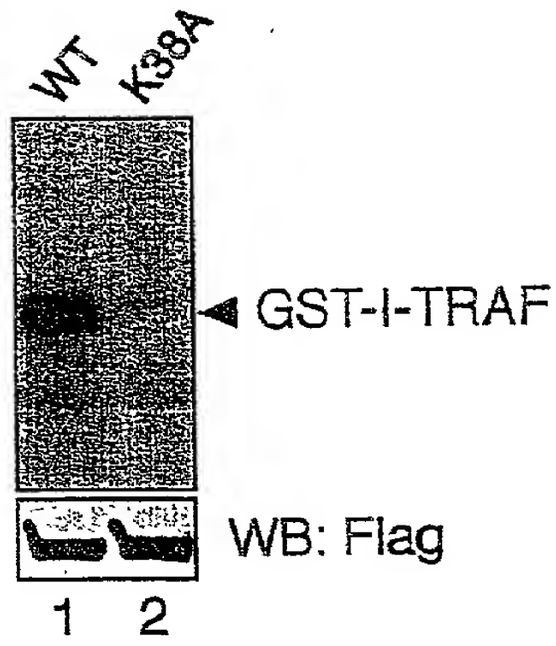


Figure 15

Declaration and Power of Attorney for Patent Application
English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

IDENTIFICATION OF NOVEL SUBSTRATE I-TRAF OF IKK-i KINASE

the specification of which

(check one)

[ ] is attached hereto.
[X] was filed on June 24, 2000 as United States Application No. or PCT Application No. 09/582,397
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
10-304085/1998 (Number)	Japan (Country)	October 26, 1998 (Day/Month/Year Filed)	[ ]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	[ ]
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	[ ]

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. Section 120 of the United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark office all information known to me to be material to patentability as defined in Title 37, C.F.C., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/JP99/05916	October 26, 2000	Pending
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Linda M. Buckley	Reg. No. 31,003	David E. Tucker	Reg. No. 27,840
Peter J. Manus	Reg. No. 26,766	Lisa Swiszcz Hazzard	Reg. No. 44,368
Peter F. Corless	Reg. No. 33,860	George W. Hartnell	Reg. No. 42,639
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